

ACTA PATHOLOGICA ET MICROBIOLOGICA SCANDINAVICA

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The Hæmum Centre Aarhus, Denmark (head professor S. Hansen MD) and
The Institute of Cancer Research Aarhus, Denmark (head J. Bichel MD)

SOME INVESTIGATIONS ON THE MECHANISM OF THE LEUCOPENIA PRODUCED BY VINCOBLASTINE

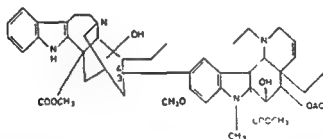
2 The Effect of Vincoblastine on Cells in Tissue Culture and in Vivo

By

J. BICHSEL

Received 15.11.66

According to Peckolt (1910) an extract of the periwinkle *Vinca rosea* Linn. was used therapeutically early in this century by the Indians in Brazil against scurvy, toothache, persisting haemorrhage, and slowly healing ulcers. However, as far as the author has found, no beneficial effect from this treatment has been reported. Furthermore, extracts of *Vinca rosea* have been used for diabetes in the Philippines and in Jamaica. The effect on the level of the blood sugar was studied simultaneously in the U.S. and in Canada, but no influence could be demonstrated. During those experiments it was found that the crude extract had an effect on the bone marrow and the white blood cell picture. In moderate doses it gave rise to a reversible leucopenia. Several alkaloids were then isolated. The first was vincoblastine (Velleb[®] Lilly) (vinblastine sulphate) which was found to be a dimeric alkaloid containing both indole and dihydroindole moieties (Corman *et al.* 1959; Neuss *et al.* 1958). The structural formula was probably



CATHARANTHINE

VINDOLINE

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Besides causing leucopenia vincoblastine also exerted an inhibiting effect on the growth of several animal tumours in mice and rats. This was shown by *Cutts, Beer & Noble* (1957) and later confirmed by many other investigators.

The problems concerning the pathogenesis of the mechanism of the leucopenia have been outlined in the introduction to the first paper of this series. Before the experimental investigations are described a brief report of what is known about the effect of vincoblastine on cells *in vivo* and *in vitro* will be given.

It is generally agreed that in tissue culture and also *in vivo* vincoblastine acts on the cells as a typical spindle poison (*Hampel & Gerhartz* 1962, 1964a, 1964b; *Cardinali et al* 1961; *Vaitkevicius* 1962; *Soldati & Gaetani* 1961; *Palmer et al* 1960) (cells from human monocytic leucemia) *Walker & Wright* 1962). Most authors describe the effect as completely similar to that of colchicine i.e. it produces metaphase arrest (c mitosis). A few authors are of the opinion that vincoblastine acts on the cell nuclei at an earlier stage of the cell division than colchicine. *Hampel & Gerhartz* describe e.g. early prophase disturbances and also claim chromosomal changes to be different from those produced by colchicine. According to those authors vincoblastine does not produce so pronounced exploded mitoses as colchicine and the pyknotic chromosomes have a greater tendency to fuse and form large pyknotic areas. *Cardinali et al* mention that *Cutts* has not observed any inhibition in the prophase but they find the inhibition of prophase in human bone marrow as well as in leucemic cells from leucemia I 1210. In cells regenerated after vincoblastine influence the nucleus will often be abnormal multilobulated and now and then the cells may contain many nuclei.

It is mentioned several times that the action of colchicine differs from that of vincoblastine i.e. that contrary to colchicine vincoblastine acts on the cells from the golden hamster (*Orsini et al* 1961). However this is probably not quite correct as colchicine acts on hamster cells as a spindle poison but the concentrations must be rather high.

Besides being a spindle poison vincoblastine is also an antimetabolite as several of its effects i.e. its action on the spindle may be prevented by certain amino acids as glutamic acid, tryptophan, α -keto glutaric acid and also by Co enzyme A (*Johnson et al* 1960). It must however be remembered that the effect of colchicine also may be prevented at least by meso inositol (*Murray et al* 1951).

The effect of vincoblastine seems to be the same *in vivo* and *in vitro* anyway as far as morphological alterations are concerned. Injections of vincoblastine to normal animals (mice, rats, dogs, hamsters) will produce a mitotic arrest. This is most pronounced in the granulopoietic tissue in the bone marrow especially in the myelocytes.

Some of the most extensive studies of the effect of vincoblastine on cells in tissue culture of bone marrow and also of the effect on cells

from leucemia I 1210 and Ehrlich ascites tumours have been done by Cuts (1961). He found that after a single injection of vincoblastine the number of mitoses increased progressively and reached a maximum after 12 hours where after the number decreased. After 48 hours the number of metaphases was still somewhat increased. In tumours the number of mitoses decreased to normal values more rapidly than in normal tissue. The arrested mitoses occurred very soon after the injection and in most animals they reached a peak in the 12th to the 16th hour in some animals already in the 8th hour. The reconstruction of the nuclei might result in deformities of the nucleus as described by Carlini *et al* (1961) but also in giant nuclei. During that process or during the production of multilobulated nuclei an exclusion of one or more chromosomes might occur. Cuts did not observe chromosome breaks as those regularly produced by alkylating agents. Cuts also demonstrated that the administration of glutamic acid just prior to vincoblastine reduced the number of arrested mitoses. His investigations were done on Swiss mice bearing an ascites tumour. Cuts compared the effect of colchicine and vincoblastine and although he emphasized that vincoblastine is a typical mitotic poison the degree of inhibition was more lasting and complete than that produced by colchicine. According to Cuts a weak concentration of vincoblastine will inhibit the formation of the spindle while strong concentrations also affect the chromosomes directly.

Valketicus (1962) found that the mitotic arrest will appear in tumours which may be inhibited by vincoblastine as well as in vincoblastine resistant tumours. Consequently it is hardly this effect which causes tumour inhibition.

In conclusion it will appear that vincoblastine is a spindle poison which probably does not act quite like colchicine. It is also stated that vincoblastine is an antimetabolite as several of its effects may be prevented by different amino acids.

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Between 1955 and 1962 the Department of Pathology of the I and II Clinics of Obstetrics and Gynecology Helsinki University Central Hospital examined a total of 23 histological specimens which could be diagnosed as mesonephric carcinoma. 12 of the specimens of the present material were collected from the clinics referred to while 11 specimens were sent in by provincial hospitals. Supplementary information on the patients was obtained from the attending doctors and the patients themselves.

The histological specimens were fixed in 10 per cent formalin for one to three days and cut with a microtome into sections 5 to 6 μ thick. The sections were stained by the haematoxylin van Gieson method. As the purpose of the investigation was only to show the diagnostic possibilities by the aid of common routine histologic examination no attempt to differentiate the varying types of carcinoma by means of special staining methods was performed in this connection.

The average age of the patients concerned was 51 years, the oldest being 84 and the youngest 36 years old. A more detailed presentation of the cases is given in Table 1. In 20 cases the tumour was situated in the ovary, in 2 in the cervix uteri and in 1 in the wall of the vagina. In the literature only 81 cases have been reported in which the mesonephric carcinoma was situated in the area of the cervix uteri (1 and 11). Mesonephric carcinoma with the exception of cyst like forms is also rare in the area of the vagina (1).

When microscopic preparations made from the tumour specimens were examined attention was paid not only to the characteristic clear cell changes (Figs 1, 2, 3) but also to structures denoting a possible connection with the serous cystadenoma tumour type (Fig. 4) as well as to endometrioid traits (Fig. 5). Besides the clear cell pattern distinct papillary structures were observed in 12 cases, smaller cystic formations in 11 cases and epithelium of serous type in 7 cases. In 8 cases the endometrioid structure predominated (cases 10, 16 and 20) and in 5 cases endometrioid structures occurred alongside clear cell changes (cases 5, 9, 13, 14 and 18).

Table 1 also gives the methods and results of treatment. In all but three cases the treatment was operative. In most cases surgery was supplemented with X-ray therapy. Two cases were treated with radioactive gold and in only one case were cytostatics given. In two cases X-ray therapy alone was given and in one (case 12) the patient refused all treatment. Four patients have survived five years without recurrence (case 4, 11, 15 and 22), one (case 19) two and a half years and one (case 23) one and a half years. One patient died as the result of an accident two years and 10 months after treatment (case 7) and one patient (case 17) died 4 years and 8 months after treatment, the cause of death being carcinoma of the breast.



Fig. 1

Fig. 1 Clear cell carcinoma with papillary architecture, stained with hematoxylin and van Gieson $\times 100$

Fig. 2 Adenomatous transformation of clear cells, stained with hematoxylin and van Gieson $\times 200$

TABLE 1

Summary of 23 Clear Cell Carcinoma

No	Age	Locality of tumor	Histol type of tumor beside clear cell structure	+	tec	Methods of treatment	Follow up
1	45	both ovaries	serous cystic papill	+	tec	hysterect bil s o ect x ray	died in 4 mos
2	49	ovary + ca corp ut	serous cystic papill	+	tec	Ra appl x ray	died in 13 mos
3	39	ovary	serous cystic papill	+	tec	sv amput bil s o ect x ray	died in 41 mos
4	49	ovary	serous cystic papill	+	tec	sv amput bil s o ect x ray	alive (7 years)
5	51	ovary	endometr	+	tec	sv amput bil s o ect x ray	died in 9 mos
6	55	ovary	serous cystic papill	+	tec	sv amput bil s o ect x ray	died in 34 mos
7	40	ovary	serous cystic papill	+	tec	sv amput bil s o ect x ray	died in 34 mos
8	36	ovary	papill endometr	+	tec	sv amput bil s o ect x ray	died in 4 mos
9	54	ovary	cystic papill endometr	+	tec	sv amput bil s o ect x ray	died in 4 mos
10	41	ovary	endometr	+	tec	hysterect bil s o ect x ray	died in 14 mos
11	48	ovary	serous cystic papill	+	tec	hysterect bil s o ect x ray	died in 16 mos
12	84	cervix uteri	serous cystic papill	+	tec	hysterect bil s o ect x ray	alive (9 years)
13	36	ovary + ca recti	cystic endometr	+	tec	nothing	died in 12 mos
14	47	ovary	cystic endometr	+	tec	co ect sin x ray	died in 9 mos
15	50	ovary	papill	+	tec	co ect dx x ray	died in 5 mos
16	39	vagina	endometr	+	tec	hysterect bil s o ect x ray	alive (8 years)
17	56	cervix uteri + ca mammae	solid clear-cell ca	+	tec	exstirp cystic vagin	died in 8 mos
18	44	ovary	endometr	+	tec	Ra appl x ray	died in 56 mos
19	77	ovary	endometr	+	tec	hysterect bil s o ect Ra appl x ray	died in 3 mos
20	36	ovary	endometr	+	tec	sv amput bil s o ect x ray	alive (2 1/2 years)
21	69	ovary	cystic	+	tec	sv amput bil s o ect	died in 3 mos
22	56	ovary	endometr papill	+	tec	hysterect bil s o ect x ray	died in 3 mos
23	69	ovary		+	tec	sv amput bil s o ect x ray	alive (8 years)
						sv amput bil s o ect x ray	alive (1 1/2 years)

tec = typical clear cell carcinoma



Fig. 3

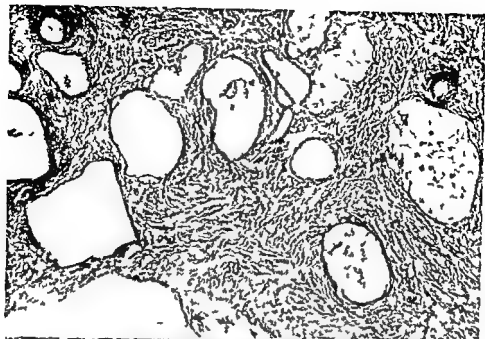
Chiefly manifestly. Histological part of clear cell carcinoma (case 19) Haem. + van Gieson
 $\times 700$

DISCUSSION

The tumours of the present series are all of clear cell type to which the term mesonephric carcinoma possibly could be applied (9).

Our cases 12, 16 and 17 are indisputable insofar that besides being of characteristic histological structure they originate from paravaginal tissue i.e. from an area in which there can hardly be any question of another type of histogenesis.

With regard to the ovarian tumours however the histogenesis is perhaps not so firmly established. For besides tumours arising from mesonephric vestiges there occur in the ovaries two other forms of carcinoma which may also be of clear cell type and distinguish between these and true mesonephric carcinoma may thus prove difficult and even impossible. One of these carcinomas is the malignant serous papillary cystadenoma. Though our series does not comprise a single grossly cystic ovarian tumour multiple multicystic formations were found in several cases as well as histological features pointing towards this type of tumour. Another tumour which may occasionally occur either partly or entirely as a clear cell type tumour is adenocarcinoma originating from ovarian endometriosis. It has been established that of this endometrioid carcinoma a clear cell type variant may occur (10). It is highly probable that endometrioid carcinoma and serous cystic



Figs 4-5

Fig 4 Structures typical of adenofibroma. *Ca e II* Haem + van Gieson $\times 100$
Fig 5 Carcinoma with partly endometrioid partly clear cell structures. Haem +
 van Gieson $\times 150$

noma of the ovary are histogenetically closely related (4). Thus our series includes cases (1-9, 10, 13, 14) in which areas of endometrioid type of varying size were seen. Consequently in our opinion which agrees with the statements of Willis (10) and Haynes & Taylor (2) a definite histogenetic diagnosis with regard to the clear cell carcinoma occurring in the ovary cannot be made for the present. Without disputing the possibility that ovarian tumours of purely clear cell type may actually originate from mesonephric remnants we still think that the two above mentioned possibilities must be considered.

Our present knowledge does not allow us in any way to try all cases to determine with full certainty the histogenesis of the ovarian carcinoma in question.

SUMMARY

The authors present 23 mesonephric carcinomas, 20 of which were situated in the ovary, 2 in the cervix uteri and 1 in the wall of the vagina. With regard to the histogenesis of ovarian carcinoma there is at any rate in some cases some uncertainty, since malignant serous cystadenoma papilliferum and endometrioid carcinoma may occur as clear cell types.

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EXPERIMENTAL INVESTIGATION ON INFLUENCE OF HYDROCORTISONE ON SPREAD OF TRANSPLANTED ROUS RAT SARCOMA IN SYNGENEIC TUMOUR HOST SYSTEM

By

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Received 18 x 66

Experimental investigations on the effect of exogenous factors on tumour spread have given controversial results which may be due to differences in methods of treatment but also to host factors or unknown intrinsic characteristics of the various tumours used (Surveys Cole *et al* 1961 Nilsen 1962)

In an earlier investigation it was shown that hydrocortisone stimulated the spread of the transplantable Rous rat sarcoma in an allogenic tumour host system (Saldeen 1963) The purpose of the present investigation was to study the effect of hydrocortisone on the spread of the rat sarcoma under the same experimental conditions in a syngeneic tumour host system

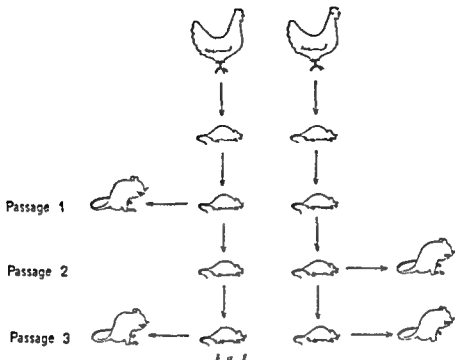
MATERIAL AND METHODS

Animals—White rats of a strain inbred since several years were used It was obtained from Dr C M Rudenski at Copenhagen and during the experimental period 8 months kept in our institute by brother sister mating

Skin grafting between the same at least 1 month old rats was performed using a method described by Thomas *et al* (1961) As controls autografts and allografts were used About one month after the operation all the syngeneic grafts and autografts were still bearing while the allografts had disappeared No signs of adverse reactions or breakdown were observed in the syngeneic grafts Still 70 weeks later the grafts were distinguishable from surrounding recipient skin only by the direction of the hair follicles

Tumour The origin of Rous rat sarcoma and the growth and spread of the transplanted sarcoma has earlier been described (Ahlfors & Jonsson 1959 Saldeen 1962 1963) In the present investigation 2 tumour strains were used (Fig 1)

Strain 1—A series of inbred newborn rats were inoculated subcutaneously with 0.1 ml cell free tumour material from a chicken with Rous chicken sarcoma (Strain S R) After an interval of 21 days all the rats had cysts in the axillae and groins and after 36 days 4 of the 8 inoculated rats had palpable tumours at the site of injection Five of the rats were killed 3 months after the injection All had tumours and cysts 4 had lymph node metastases and 3 had lung metastases One of the tumours was transplanted subcutaneously to a series of newborn rats Tapes were obtained after 3 weeks and the tumour has since been carried serially in at most 7 day old rats (Fig 1) Tumour material from the first and third passages were used for the experiments



Transplantation scheme. Small rats = rats for serial transplantation.
Dig rats = rats for the experiments.

Strain A—The serial tumour originated from a series of (inbred new) inbred rat inbred subcutaneous (lv with 0.1 ml of free chicken tumour material. After 14 days all 10 rats had palpable tumours and were killed after 56 days. Two of them had lymph node metastases and one lung metastasis. One of the tumours was transplanted to 7 new inbred rats. They were killed after 16 days when 6 had tumours, 2 with lung metastases. The tumour was then serially transplanted in a total of 4 new old rats (Fig. 1). Tumour material from the serial and third passage of all strains were used for the experiments.

Characteristics of the transplantable tumour—The growth and the histological appearance of the tumour did not differ from that earlier described in all genetic tumour test system (Hilstrom & Jensen 1962; Afton 1962, 1963). The individual variations were not significant in syngeneic than in allogeneic system.

Preparation of tumour suspension—A slight modification (Afton 1965) of a trypsin-DNAase method described by Matten & Hurl (1961) was used. The tumour cells were counted in a Buerker haemocytometer after dilution 1:100 in physiological saline containing 0.01 per cent gelatin. Only uncultured (cloning) cells were counted.

RESULTS

Intramuscular tumours in the thigh The material consisted of 48 female rats weighing about 170 g, and divided into 2 equal groups. They were inoculated intramuscularly in the left thigh with 10^7 living tumour cells 1 month 2 days before the injection of tumour until the end of the experiment. 24 of the rats were treated with 20 mg hydrocortisone acetate (Hydrocortil Pharms) subcutaneously in the right thigh while the other 24 rats were treated with NaCl. The rats were killed

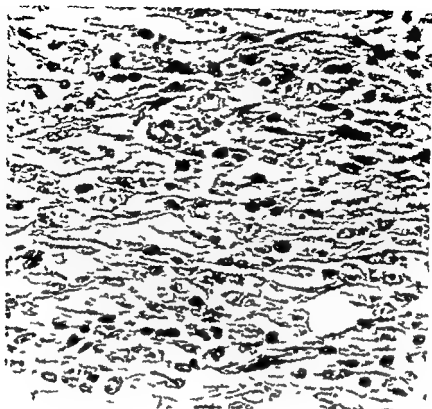


Fig. 2a

Rous rat sarcoma. Un-treated rats

3 weeks after the tumour injection. All had tumours at the site of injection. The average tumour volumes ($V = 0.5236 d^3$ d mean diameter of the tumour) were 20.3 cm^3 for the hydrocortisone treated rats and 23.8 cm^3 for the controls. The difference was not significant. The average carcass weight (without tumour) was 180 g in the hydrocortisone group 235 g in the control group. The difference was significant. No certain histological differences could be found in the structure of the primary tumours in the 2 groups. In 11 of the hydrocortisone treated rats, however, there was a slight tendency towards disintegration of the stroma with loosening of the tumour cells (Fig. 2).

One of the control rats showed metastases in the lumbar lymph nodes whereas 7 of the hydrocortisone treated rats had metastases in the regional nodes (lumbar or inguinal). Three other hydrocortisone rats showed metastases also in more remote lymph nodes (renal, cisternal or axillar) and in the lungs.

Treatment with hydrocortisone thus resulted in an increased spread of the intramuscular tumours in the thigh to lymph nodes and lungs.

Intravenous injection of tumour cells—Nineteen female rats weighing about 200 g were inoculated intravenously into a tail vein with

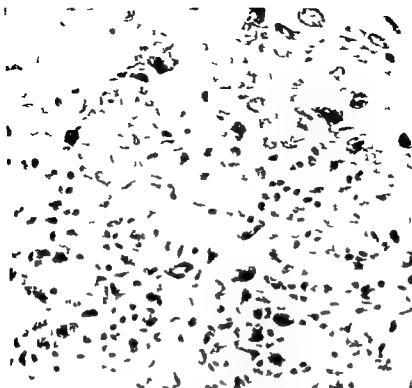


Fig 9b

Rous rat sarcoma Hydrocortisone treated rat Disintegration of stroma and loosening of tumour cells.

5×10^5 living tumour cells Eight of them were treated on 2 days before the injection of the tumour and throughout the experiment with 2.5 mg hydrocortisone daily The other 11 rats served as controls The rats were killed on the 15th day after the tumour injection The weight of the control rats varied between 220–250 g (average 230 g) that of the hydrocortisone treated animals between 160–190 g (average 170 g)

Seven of the hydrocortisone treated rats and one of the controls had widespread pulmonary tumours whereas the other rats showed no pulmonary tumours No rats had tumours outside the lungs The pulmonary tumours often showed large central necroses

Treatment with hydrocortisone thus resulted in an increased frequency of metastases in the lungs following intravenous injection of tumour cells

DISCUSSION

There was no general difference in respect of growth and spread of Rous rat sarcoma in untreated rats in syngeneic tumour host system compared with that in allogeneic system

As to the effect of hydrocortisone in the allogeneic system (Saldeen 1963) 18 of the 19 treated rats against 2 of the 18 controls had lung metastases after intravenous injection of tumour cells. In rats with intramuscular tumours 1 of 10 had metastases to the regional lymph nodes only while the other 9 rats had metastases to more remote lymph nodes also. One of 10 control rats had metastases in regional lymph nodes.

A comparison of the effect of hydrocortisone on the spread of allogeneic (Saldeen 1963) and syngeneic Rous rat sarcoma thus speaks for hydrocortisone having almost identical effect in the 2 systems after intravenous injection of tumour cells but a more pronounced effect in the allogeneic system than in the syngeneic on the spread of intramuscular tumours. This may be due to small differences (not morphologically verified) between the tumour strains used in the two experiments *e.g.* in the present investigation only very early tumour passages were used. It seems not probable that the hydrocortisone effect in the allogeneic system occurred via an immunological enhancement since Kaliss *et al.* (1954) found no effect of cortisone on metastases of allogeneic tumours in mice subjected to immunological enhancement.

Also in the syngeneic Rous rat sarcoma system there are lack of complete genetical identity between tumour and host since Rous sarcoma of the strain used possesses common tumour specific antigen(s) (Jonsson 1966).

The hydrocortisone may possibly act by modifying the discrepancy between tumour and host.

However it seems probable that hydrocortisone also has a direct effect on the tumour spread in addition to its possible indirect effect via the influence on tumour host relationship in transplantable systems. It have been shown earlier that cortisone promotes the spread of human tumours (Iversen & Hjort 1958, Hartman & Sherlock 1961).

There are at least seven possible points of attack of an exogenous factor on the lymphatic spread (Fig. 3). The exogenous agent may act on

- 1) Primary tumour *e.g.* by altering the mutual cohesiveness
- 2) Tumour host interzone *e.g.* by modifying the stroma reaction
- 3) Individual cancer cells *e.g.* by changing amoeboid activity or surface properties
- 4) Vascular invasion *e.g.* by modifying capillary permeability
- 5) Vascular endothelium *e.g.* by altering the endothelial stickiness (Zeidman 1962)
- 6) Lymph *e.g.* by changing the coagulability
- 7) R.E.S. *e.g.* by modifying the phagocytic activity of the reticular cells in the cortical sinus

The present investigation speaks in favour of hydrocortisone acting on the first (Fig. 2b) point. Hydrocortisone is however also known to

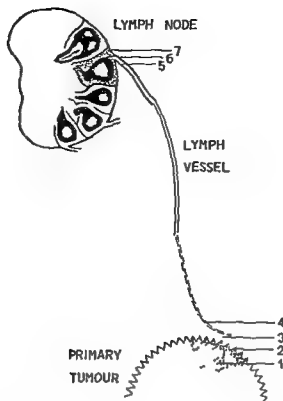


Fig 3

Possible points of attack of an exogenous factor on lymphatic spread. See text.

reduce the function of RES and treatment by this drug results in involution of the lymphatic tissue. It thus probably also promotes spread by acting on the last point.

No histological differences were seen in the tumour host zone between hydrocortisone treated rats and controls. Neither were any differences seen between the morphological picture of individual cancer cells in the lymph nodes of cortisone treated rats and controls. This however of course does not exclude that hydrocortisone may modify the properties of the cells.

Hydrocortisone is not known to increase capillary permeability.

The haematogenous spread of the tumour may have been influenced by the effect of hydrocortisone on the stickiness of the vascular endothelium (Zeidman 1962) or the blood coagulability in the pulmonary vessels. Tumour cells injected intravenously into a tail vein can pass the lungs in untreated animals (Saldeen 1963) and are possibly trapped to a greater percentage in the lungs of hydrocortisone treated rats. As almost all tumour cells are trapped by the lymph node sinus already in untreated rats possible similar effect (Saldeen 1963) of hydrocortisone

on stickiness and lymph coagulability in the lymph node sinus are probably of minor importance

SUMMARY

Treatment with hydrocortisone promoted tumour spread in rats with early passages of transplantable Rous rat sarcoma in a highly inbred brother sister mated strain. It seems most probable that hydrocortisone promotes the lymphatic spread of the tumour by acting on the primary tumour and on the RLS.

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EFFECTS OF NITROGEN MUSTARD ON THE DEVELOPMENT OF EXPERIMENTAL AMYLOIDOSIS IN MICE

By
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Received Jan 66

An accelerating effect of nitrogen mustard on the formation of amyloid deposits was demonstrated by Teitlum in 1954. In his experiments mice previously treated with casein for four weeks rapidly developed a wide spread amyloidosis after three doses of 2.5-5 mg/kg nitrogen mustard each second day while no or only a slight increase occurred in the controls. Earlier investigations by the same author had showed a similar accelerating effect of cortisone and ACTH (Teitlum 1952). In the same way γ irradiation has been shown to be able to promote amyloid formation in mice (Christensen & Hjort 1959 1960). A common feature of all these experiments was the observation that an acceleration of the amyloidogenic process depended on the time of administration of the proper accelerating agent. Only when applied late in the experiment—that is near the end of the primary pyroninophilic phase or in the second amyloid phase could acceleration be constantly achieved. On the other hand when cortisone was given before or simultaneously with the amyloidogenic treatment—that is during the initial pyroninophilic phase formation of amyloid in mice was inhibited or in some cases even prevented in spite of 8 weeks of casein treatment. This inhibition was found to involve a suppression of the cellular proliferations showing cytoplasmic pyroninophilia (Christensen 1961). These seemingly contradictory effects of cortisone support the two phase theory of local amyloid formation (Teitlum 1964) and it has been anticipated that other agents may exhibit the same pattern of inhibition acceleration depending on dose and time of administration during amyloid induction. Recently heterologous antilymphocytic serum has proved effective in preventing amyloid formation in casein treated mice when applied during the initial pyroninophilic phase (Ranlov 1967). In the following the effects of nitrogen mustard on amyloid formation in casein treated mice will be shown to vary depending on the time of administration.

This investigation was supported by The Danish League Against Rheumatism

MATERIAL AND METHODS

ST/a mice of equal sex distribution 3-4 months of age were used after randomization. They were divided as follows:

Group I

15 mice were treated for 4 weeks with daily subcutaneous injections 5 times a week of 0.5 ml 5 per cent sodium caseinate in 0.25 per cent NaOH—a total of 20 casein injections. Simultaneously nitrogen mustard was administered every second day (Monday–Wednesday–Friday) for 4 weeks 0.02 mg nitrogen mustard (Frasol®) in 0.2 ml normal saline was injected subcutaneously in the back—a total of 12 injections.

Group II

15 mice were casein treated as described above. In addition 12 subcutaneous injections of 0.2 ml normal saline were given as described for nitrogen mustard above.

Group III

12 mice were treated essentially as those of group II. At the end of 4 weeks a course of 3 nitrogen mustard injections was administered over 5 days (each dose 0.02 mg/0.2 ml normal saline).

Group IV

16 mice were treated for 4 weeks with daily subcutaneous injections 5 times a week of 0.5 ml normal saline—a total of 20 injections. Simultaneously nitrogen mustard was administered as described for group I.

The day after the last injections all animals were killed with ether inhalation and the following tissues were fixed in neutral formalin: spleen, liver, kidney, adrenal, ilium, lymph node, lung and thymus. Paraffin embedded sections were stained with methyl green picroin, haematoxylin-eosin, alkaline Congo red and the PAS technique. The grading of amyloidosis in individual animals was a subjective estimation based on the broadness of the regular per follicular amyloid rings in the spleens as described by Christensen & Hjort (1959).

TABLE 1

The Effect of Nitrogen Mustard on Amyloid Formation in Various Groups of Casein Treated and Control Mice

Group	Treatment	Number of mice developing amyloidosis/total	Mean degree of amyloidosis
I	casein + nitrogen mustard	11/15	++
II	casein + saline	15/15	++++
III	casein + saline followed by nitrogen mustard	12/12	+++++
IV	saline + nitrogen mustard	1/16	0

RESULTS

The main results of the present experiment are outlined in Table 1. It appears that both the incidence and severity of amyloidosis were re-

duced in casein treated mice simultaneously treated with nitrogen mustard (group I) as compared to mice casein treated alone (group II). In contrast mice which after the completion of 4 weeks of casein treatment received a short course of nitrogen mustard treatment (group III) showed an increase in the mean degree of amyloidosis as compared to mice casein treated alone. That nitrogen mustard alone can not be considered amyloidogenic is evident from the figures of group IV.

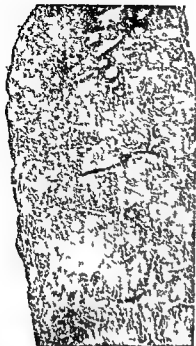
Among the animals of group I 4 mice failed to develop amyloid at all in spite of 4 weeks of casein treatment. In these 4 mice the dominating histological feature was a marked depletion of lymphoid tissue especially pronounced in the spleen (Fig 1) which also showed a rather marked pyroninophilia. P.A.S. staining reticular cells were seldomly found. In the remaining 11 mice splenic amyloid was found typically arranged around the depleted Malpighian corpuscles (Fig 2) in amounts ranging from grade ++ to grade +++ (mean ++). Only a few of the animals of this group had recognizable amyloid deposits in liver or kidneys. In contrast mice of group II which were casein treated for 4 weeks without receiving nitrogen mustard all exhibited a heavy splenic amyloidosis (Fig 3) ranging from grade +++ to grade ++++ (mean ++++). In addition all of these animals showed hepatic and renal amyloid deposits. An even more pronounced amyloidosis was found among the animals of group III which after 4 weeks of casein received a short course of nitrogen mustard treatment. In comparison only 1 out of 16 mice (group IV) nitrogen mustard treated for 4 weeks showed splenic amyloid and only to a very small extent. The remaining 15 mice had no detectable signs of amyloidosis, the predominant lesion being a rather heavy lymphoid depletion (Fig 4).

In group I pyroninophilia of splenic reticular cells was marked especially in the perifollicular areas. In the groups II and III this pyroninophilia was found to be less pronounced while P.A.S. positive reticular cells were frequently found in the border zones of the amyloid deposits. In group IV pyroninophilia was modest.

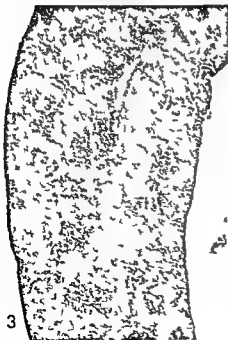
The amyloid was identified by its pink staining with Congo red which under crossed polars showed a brilliant green birefringence. It was slightly eosinophilic and stained light purple with the P.A.S. stain except for the small new formed amyloid deposits which stained vividly purple.

DISCUSSION

The results of the present experiment indicate that nitrogen mustard when applied early during the course of an amyloidogenic treatment may inhibit and in some cases prevent amyloid formation. Its mechanism of action in these respects is probably akin to the similar amyloidosis inhibiting effect of cortisone (Christensen 1961) and of anti lymphocytic serum (Ranlov 1967) when applied from the beginning of the casein treatment. In all these cases the effect most probably



2



3



4

relies on a suppression of the immune response which is believed to initiate the amyloidogenic process and which reflects itself in the pyroninophilia so characteristic of the reticular tissues (especially the spleen) during the initial preamyloid phase of experimental amyloidosis. Both in the experiments of *Christensen* (1961) using cortisone and in the present investigation a suppression or postponing of the primary pyroninophilic phase of amyloid formation was evident. However the present results show a somewhat less pronounced amyloidosis inhibiting effect of nitrogen mustard than that of cortisone and antilymphocytic serum. This is believed to be a question of dosage. If a sufficient suppression of the immune apparatus is not achieved the result may actually be an enhancement of the amyloidosis. This is illustrated by the acceleration of casein induced amyloidosis in mice subjected to adult thymectomy prior to the casein treatment (*Kanlov* 1966 a) or to sublethal irradiation (*Turunen & Teir* 1961).

The various experiments in which cortisone and nitrogen mustard have been shown to inhibit amyloid formation when applied *early* during the first few weeks of casein treatment viewed together with the experiments in which the same two compounds applied *late* in the course of an amyloidogenic treatment have been shown to enhance amyloid formation (*Teitum* 1952, 1954) are by no means conflicting. On the contrary they offer a perfect illustration of the two phase cellular theory of local amyloid formation (*Teitum* 1964). The initial preamyloid phase characterized by proliferation of pyroninophilic protein synthesizing cells is the presupposition of the second amyloid phase which is characterized by the functional break down of protein synthesizing cells in the face of a prolonged antigenic stimulation of the mesenchyme. The result is the deposition *in situ* of abnormal proteins (amyloid). Inhibition of the former phase precludes the development of the latter. Inhibition of the latter will promote the imminent break down thereby enhancing the subsequent amyloid formation.

Figs 1-4

- Fig 1 Section of spleen from mouse after 4 weeks of combined treatment with casein and nitrogen mustard. The spleen shows marked lymphoid depletion but no amyloid. Haematoxylin eosin $\times 35$.
- Fig 2 Section of spleen from mouse after 4 weeks of combined treatment with casein and nitrogen mustard. The spleen shows lymphoid depletion and small amounts of amyloid arranged in the perifollicular regions (Grade ++). Haematoxylin eosin $\times 35$.
- Fig 3 Section of spleen from mouse after 4 weeks of casein treatment showing grade +++ amyloidosis. Haematoxylin eosin $\times 3$.
- Fig 4 Section of spleen from mouse after 4 weeks treatment with nitrogen mustard. a. Lymphoid depletion. b. amyloid. Haematoxylin eosin $\times 1$.

SUMMARY

It is known that cortisone may inhibit amyloid formation in mice when administered prior to and during the first few weeks of casein treatment while it may enhance the amyloidosis when given late in the course of an amyloidogenic treatment. A similar amyloid enhancing effect of nitrogen mustard has been described.

In order to evaluate the effect of the time of administration nitrogen mustard was injected into groups of mice at various stages during casein treatment. It appeared that both the incidence and severity of amyloidosis was reduced in casein treated mice simultaneously treated with nitrogen mustard from the beginning of the experiment as compared to mice casein treated alone.

The results are discussed in light of the two phase cellular theory of local amyloid secretion.

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THE FUNCTION OF THE ADRENAL CORTEX IN MICE WITH SPONTANEOUS ADRENOCORTICAL LIPID DEPLETION

By

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A spontaneously occurring lipid depletion of the adrenal cortex in mice of the AhR/O strain has been described by *Arnesen* (1955 and 1956). The lipid depletion coincides with sexual maturation in both sexes and is due to the effect of a single recessive gene the adrenocortical lipid depletion gene. Prepubertal gonadectomy prevents the lipid depletion from taking place. Hybridization experiments were carried out between the high leukemia AhR/O and the non leukemic WLO strain. One of the resulting hybrid lines designated CS has maintained the adrenocortical lipid depletion and the inherited susceptibility to lymphatic leukemia derived from AhR/O strain however with a slightly reduced incidence of leukemia (*Arnesen* 1963 and 1964). The lipid content of the adrenal cortex can easily be estimated by inspection of the glands through laparotomy during life or by autopsy. The normal lipid rich adrenal is bright yellow whereas the lipid depleted adrenal is red. The origin and characteristics of the WLO strain which has been used for comparative studies in the present work are described by *Kreyberg* (1952).

It is not possible to make any generalization as to a correlation between the amount of lipid as seen histologically in frozen sections stained with Sudan III and the functional state of the gland. An adrenocortical lipid depletion as such is compatible both with hyperfunction and with hypofunction of the adrenal cortex. *Velcalf* (1960) who confirmed the existence of the spontaneous adrenocortical lipid depletion described by *Arnesen* (1956) in the AhR strain stated that the adrenal in AhR mice is hypofunctional with respect to production

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Fig 1
CS male 12 weeks old Adrenal gland Spontaneous cortical lipid depletion
Sudan III $\times 43$



Fig 2
W10 male 12 weeks old Adrenal gland Normal cortical lipid pattern
Sudan III $\times 43$

of lymphocytolytic steroids. Levine & Treiman (1964) found a decreased response to stress in AHR mice. The authors observed an impaired rise of plasma corticosterone levels in AHR mice after an electric shock when compared with control animals. Arnesen (1964b) on the other hand found that in mice with spontaneous adrenocortical lipid depletion treatment with cortisone reversed the lipid pattern and the cytological details of the cortical (fasciculata) cells to conditions found in most strains of mice. These observations suggested that the spontaneous lipid depletion might be the result of increased pituitary stimulation of the adrenal cortex. The author stressed however that definite conclusions in this respect were not yet possible.

The purpose of the present study is to investigate the adrenocortical function in male CS mice and in males of the W10 strain. In Figs 1

and 2 the characteristic lipid pattern of the two strains are depicted Fig 2 representing the normal one. We were primarily interested in measuring the plasma corticosterone levels in ACTH stimulated animals. Furthermore it was aimed to determine the corticosterone content of the adrenals in resting animals and to carry out *in vitro* studies on the production of corticosteroids in adrenal glands incubated with ACTH.

MATERIAL AND METHODS

Animals

Male mice of the CS and WLO strains were used. In order to procure CS mice for the experiments all matings of all animals since 1963 have been arranged by the author in the Department of Pathology, Ullevål Hospital. The strain has been maintained by brother-to-sister matings. Since 1962 in the same institution the author has arranged all matings of WLO animals to raise a sufficient number of WLO males for the present study. The strain has been maintained by brother-to-sister matings or father-to-daughter matings if a brother was lacking.

As a routine the young mice were separated from their parents and littermates at weaning during their 4th week. The males were kept isolated in single cages measuring $12 \times 29 \times 12$ cm with opaque plastic walls to their 12th week at which time they were killed. When plasma corticosterone was measured in 3-week-old prepubertal mice the animals were not removed from the breeding cages until the day of experiment. However when the study included investigations of adrenals from prepubertal male the animals were isolated from the age of 3 weeks. The mice were then kept in single cages as outlined above for another week before the experiments were carried out.

All details concerning the conditions in the animal house, the housing and feeding of the mice are given elsewhere (Solem 1966). The mean weights of the animals are given with the results (see the tables).

All possible precautions were taken that CS mice and controls were raised and housed under identical conditions. During the various procedures included in the investigations the animals were handled in the same way and great care was taken that every step of each experiment should be identical for the CS and WLO mice. In all experiments where blood or adrenal tissue was collected from CS mice it was confirmed by inspection that the gross colour of the adrenal glands corresponded to the age of the animals. In 12-week-old CS males the lipid depleted adrenals have a deep reddish colour resembling the colour of kidney tissue without a trace of yellow as previously described by A. Jensen (1956) in the AKR/O mouse. Plasma and adrenals from young CS males 3-4 weeks of age were only included in the present study when gross inspection had shown that the adrenals had the usual bright yellow colour found in most normal strains of mice indicating that lipid depletion had not yet taken place. As a rare finding the colour of the adrenal glands in these young CS males was appraised to be doubtful or even reddish and such animals were excluded from the experiments.

Experimental Procedures and Agents

According to our previous experience it is extremely difficult to obtain reliable true resting values of plasma corticosterone in mice (Solem 1966). In the present study we therefore carried out a very limited number only of plasma analyses in resting CS and WLO mice 12 weeks of age. The data not included in this paper showed no significant strain difference in the plasma corticosteroids of adult males. However in several experiments plasma corticosterone was measured in blood collected from animals stimulated with ACTH. As a standard procedure 100 mU were given subcutaneously in a single injection and the animals sacrificed 60 minutes thereafter. All details of the performance of the ACTH stimulation are discussed in a previous paper (Solem 1966). Mice of the two strains were always injected with the same batch number of cortrophin.

¹ The following batch number of Actortan (Actortin) Ferring A/S - Malmö Sweden were used 63031 and 63012.

We were interested in examining a possible difference in the corticoid content of the lipid depleted adrenal glands of CS mice and the normal adrenals of WLO mice. The material for these studies was provided from 12-week-old resting males of the two strains.

The output of corticosteroids from the adrenals was investigated by *in vitro* studies. Adrenal glands from resting CS mice and controls were incubated with ACTH peptide and the amount of corticoids was measured in the incubation medium. The adrenals from the two strains were always incubated with the same batch number of ACTH peptide.³

Measurement of Plasma Corticosterone

The sampling of blood was performed by collecting arterio-venous blood in heparinized centrifuge tubes from the trunk portion of mice rapidly decapitated with a pair of scissors without anaesthesia. When blood was pooled the lipid content of the adrenals of each mouse was always estimated through inspection of the colour of the adrenals *post mortem*.

Determination of plasma corticosteroids was carried out on 0.50 ml aliquots of plasma according to a modification of the fluorometric method of Silber, Busch & Ostlapas (1958) and Cuillemin, Clayton, Smith & Lipscomb (1958) elaborated and evaluated by Solem & Brinck-Johnsen (1966). The levels of corticoids were expressed as μg corticosterone per 100 ml plasma. The 0.50 ml plasma aliquot for analysis derived from blood pooled from 2 to 3 mice. Occasionally an aliquot of 0.25 ml plasma was analysed.

In a limited number of 10 ml plasma aliquots we separated the fractions of bound and unbound corticosteroid using gel filtration. The corticosteroids in the different fractions of the eluate were determined as corticosterone with the fluorometric procedure referred to above. The gel filtrations were carried out according to the method described by De Voor *et al* (1967) and the details of the performance of the gel separation in our laboratory are presented in a preceding paper (Solem 1966).

Measurement of Adrenal Corticosterone Content

All precautions were taken to obtain the adrenal glands while the animals were in a resting state at 08.00 a.m. A procedure identical to that described in a recent study (Solem 1966) to attain true resting values of plasma corticosterone was used. The animals were killed by cervical dislocation. CS and WLO males were sacrificed alternately. The adrenals were rapidly removed, immediately frozen on dry ice and kept frozen for 24 hours. The glands were then carefully dissected at room temperature. The adrenals from 10 animals of each strain were separately pooled, weighed on an analytical balance and thoroughly disintegrated in 10 ml distilled water using a MSE Homogeniser³. To the tissue suspension was added another 10 ml distilled water which simultaneously was used for removing particles of adrenal tissue adherent to the cutter blades and washing them into the glass container. The 20 ml diluted tissue suspension in the container was then divided as exactly as possible in two equal parts and transferred to two glass stoppered centrifuge tubes and from this step on the analysis was carried out in duplicate. The procedure was the following:

The approximately 10 ml tissue suspension in each tube was extracted thrice with 15 ml methylene chloride as described in a previous paper (Solem & Brinck-Johnsen 1966). The total extract was gradually evaporated under vacuum and ultimately brought to dryness. The residue was dissolved in 2 ml petroleum ether by gentle inversion of the tube by hand 10 times. A partition with 70 per cent ethanol was performed first with 10 ml once then twice with 6 ml. Between each partition which was performed by shaking 100 times by hand the first time gently and later more vigorously, a 10 minutes centrifugation at 2000 r.p.m.⁴ was carried out. The ethanol

ACTH peptide: Lerring AB, Malm, Sweden. Batch no. 124 140 IU (intravenously) per mg.

³ Measuring and Scientific Equipment Ltd, London, England (Catalogue No. 7700). Propeller type.

⁴ g value 1050.

was evaporated under N_2 to a volume of about 15 ml and extracted thrice with 15 ml methylene chloride. The final extract was evaporated under N_2 and brought to dryness and was ready for chromatography. The chromatography was carried out as previously described (Solem & Brinck-Johnsen 1965). For computing purposes duplicates of a corticosterone standard 0.30 μg with 10 ml distilled water added and a water blank of 10 ml were carried through the whole procedure along with the adrenal tissue samples. To locate the corticosterone derived from the adrenal tissue suspension 24 μg corticosterone were simultaneously chromatographed on a vacant strip of the same paper. The further procedure was then as described previously when carrying out measurements of plasma corticosterone after chromatography (Solem & Brinck-Johnsen 1965). The amount of corticosteroids was expressed as μg corticosterone per 100 mg adrenal tissue and per pair adrenal glands.

A description of the reagents and equipment used in these procedures was presented in the paper by Solem & Brinck-Johnsen (1965).

Measurement of the Corticosterone Production by Mouse Adrenals Incubated with ACTH

The *in vitro* method described here is almost the same as that of Saffran & Shally (1955) as modified by Van der Vies (1957). Sixteen mice (8 CS and 8 WLO males) were used for each experiment. All precautions were taken to avoid exciting the animals and causing a possible activation of the pituitary adrenocortical system on the day of experiment which started at 08.00 a.m. The singly housed animals were killed one by one by rapid cervical dislocation. CS and WLO mice were sacrificed alternately. The adrenals were removed from each mouse rolled gently on a dry filter paper freed from adhering tissue carefully bisected and each half was placed on one of two pieces of tinfoil kept in a petri dish lined with moistened filter paper. The bisection was performed as accurate as possible so that two almost equal halves were cut from each adrenal. The two petri dishes one for the adrenal halves from the CS mice distributed on two tinfoil pieces and another correspondingly containing the WLO adrenal halves were kept cooled on crushed ice. At the end of distribution the four tinfoil pieces with the adrenal tissues were weighed on an analytical balance. The adrenal halves were then placed in four 50 ml glass stoppered Erlenmeyer flasks. Each flask contained 5 ml of Krebs Ringer bicarbonate glucose medium prepared according to Van der Vies (1957) and which had previously been bubbled with a mixture of 95 per cent O_2 and 5 per cent CO_2 for 10 minutes. The 4 flasks were mounted well flushed with the gas mentioned above and shaken at 38°C for one hour (preincubation period). After the preincubation period the medium from the flasks was sucked off as completely as possible and replaced with 5 ml of fresh medium. To each flask was then added 3.5 IU ACTH in a volume of 0.1 ml and the flask again well flushed with the mixture of 95 per cent O_2 - 5 per cent CO_2 . The adrenals were then shaken for two hours (incubation period). At the end of the first hour the same dose of ACTH was added to the medium making the total amount of ACTH in each flask 7 IU. After the incubation period the medium was removed to four glass stoppered centrifuge tubes labelled CS I, CS II, WLO I and WLO II. For the transfer 5 ml distilled water was used for each flask to secure the complete removal of the medium. From this step the analysis was carried out in duplicate according to the following procedure.

The medium was extracted twice with 15 ml methylene chloride as described in a preceding paper (Solem & Brinck-Johnsen 1965). The total extract was gradually evaporated under N_2 and finally brought to dryness and was then ready for chromatography. The chromatography was carried out in the same way as described earlier (Solem & Brinck-Johnsen 1965). Duplicate of a corticosterone standard 1.0 μg with 10 ml distilled water added and a water blank of 10 ml were run with the extracts of the incubation media. To one of the strips of chromatography paper were applied 24 μg corticosterone for locating the corticosterone derived from the extracts and the standard. Concerning the final procedure including elution and fluorometric measurement of the corticosterone we refer to the above mentioned publication. The amount of corticosteroid measured in the medium after 2 hours

⁵ The ACTH peptide was dissolved in 0.025 N acetic acid and diluted with saline to a concentration of 35 IU per ml. The solution was freshly prepared for each experiment.

incubation of the adrenals with ACTH was expressed as μg corticosterone per 100 mg adrenal tissue and per two adrenal glands

Statistical Methods

Differences were tested for statistical significance by means of the t-test

Precision has been assessed from pairs of measurements by means of the standard error of measurement

$$\sqrt{\frac{\sum D^2}{2n}}$$

where D is the differences and n the number of pairs

RESULTS

Plasma Corticosterone Levels after Stimulation with ACTH

Sixty minutes after subcutaneous injection of ACTH the plasma corticosterone in 12 week old male mice of the CS strain had reached a level which was distinctly lower than that observed in stimulated control animals (Table 1). The response to ACTH in 3 week old CS males was on the other hand virtually identical with that obtained in WLO male mice of the same age

TABLE 1

Plasma Corticosterone in 3- and 12-Week-Old CS and WLO Male Mice 60 Minutes after Subcutaneous Injection of 100 mU ACTH

Animals	Age (weeks)	No of samples (each a pool from 2 to 3 mice)	Plasma corticosterone (μg per 100 ml)		Standard deviation
			Mean	Range	
CS	3	10	51.7	44.9-62.3	3.6
WLO	3	10	52.0	45.1-64.8	3.6
CS	12	20	32.2	27.1-37.2	3.2
WLO	12	20	42.1	34.4-47.0	4.2

Mean weight

CS mice	3 weeks old	12.2 g (range 8.5-14.5 g)
CS mice	12 weeks old	23.9 g (range 19.5-24.5 g)
WLO mice	3 weeks old	11.3 g (range 8.0-13.0 g)
WLO mice	12 weeks old	22.1 g (range 16.5-24.0 g)

Using gel filtration we separated the fractions of bound and unbound corticosteroids in plasma pooled from 12 week old animals after ACTH stimulation. The animals had been housed isolated from weaning. Table 2 shows that the reduced response to ACTH observed in adult CS males is due to a repressed increase of both fractions of corticosterone

Adrenal Corticosterone Content

The content of corticosterone measured in the adrenal glands in resting CS males 12 weeks of age was found to be lower than that of the controls (Table 3)

TABLE 2
*Plasma Corticosterone in 19-week-old CS and W/O Male Mice 60 Minutes after Subcutaneous Injection of 100 mU ACTH
 The (corticosterone Was Measured before (Regular Procedure) and after Cel filtration All Data Represent Means of Duplicate Samples*

Experiment No	Regular procedure Plasma corticosterone (μ g per 100 ml)	Cel filtration Plasma corticosterone (μ g per 100 ml)			Recovery %
		Unbound	+ bound	total	
CS mice					
1	31.4	15.8	14.3	30.1	95.9
2	33.0	17.1	15.1	32.2	97.6
Mean	32.2	16.5	14.7	31.2	96.8
Standard deviation	1.1			1.5	
Standard error of measurement (based on the differences observed in the duplicates)	0.6	0.7	0.6		
W/O mice					
1	42.0	22.8	17.3	40.1	95.5
2	43.1	24.4	17.3	41.9	97.2
Mean	42.6	23.6	17.4	41.0	96.4
Standard deviation	0.8			1.3	
Standard error of measurement (based on the differences observed in the duplicates)	0.6	1.1	1.3		

For each experiment was used a plasma pool obtained from 10 to 15 mice

TABLE 3

Corticosterone Content in Mouse Adrenals The Animals Were 12-Week-Old CS and WLO Males Singly Housed and Resting All Data Given Represent the Means of Duplicate Samples

Experiment No	No of mice	Corticosterone content $\mu\text{g}/100 \text{ mg}$ adrenal tissue	Corticosterone content $\mu\text{g}/\text{pair}$ adrenals	Weight of adrenal pair mg
CS mice				
1	10	0.502	0.0106	2.11
2	10	0.417	0.0101	2.42
3	10	0.412	0.0091	2.21
4	10	0.398	0.0092	2.31
Mean		0.432	0.0098	2.26
Standard deviation		0.047	0.0007	
Standard error of measurement (based on the differences observed in the duplicates)		0.03	0.0007	
WLO mice†				
1	10	0.597	0.0120	2.01
2	10	0.606	0.0143	2.36
3	10	0.644	0.0139	2.16
4	10	0.555	0.0122	2.20
Mean		0.600	0.0131	2.18
Standard deviation		0.037	0.0012	
Standard error of measurement (based on the differences observed in the duplicates)		0.02	0.0005	
Mean weight CS mice 24.1 g (range 20.5-26.5 g)				
† Mean weight WLO mice 22.3 g (range 17.5-25.0 g)				

The Corticosterone Production by Mouse Adrenals Incubated with ACTH

Table 4 shows the results of the *in vitro* studies. The production of corticosterone by adrenal glands of 12 week old CS males was found to be markedly lower than by the adrenals from WLO male mice of the same age. However the corticosteroid production in adrenals from prepubertal males of both strains was observed to be within the same range (Table 5).

DISCUSSION

The lipid depletion of the adrenal cortex in mice of the CS strain is known to coincide with sexual maturation.

The observations made in the present study have not revealed any difference in the adrenocortical function of prepubertal CS males 3 to 4 weeks old and of WLO males of the same age.

TABLE 4

Corticosterone Production in vitro in Mouse Adrenals Incubated with ACTH The Animals Were 12-Week-Old CS and WLO Males Singly Housed and Resting The Adrenals Were Removed and Bisected After Preincubation the Adrenal Halves Were Incubated with ACTH for 2 Hours and the Amount of Corticosterone in the Medium Was Measured All Data Given Represent the Means of Duplicate Samples

Experiment No	No of mice	Corticosterone production μ g/ 100 mg adrenal tissue/2 hours	Corticosterone production μ g/ 2 adrenals/ 2 hours	Weight of 2 adrenals mg
CS mice				
1	8	44.19	1.71	2.73
2	8	43.03	1.10	2.55
3	8	43.14	1.26	2.93
4	8	46.93	1.41	3.01
5	8	44.35	1.13	2.54
6	8	39.67	1.03	2.60
Mean		43.54	1.19	2.73
Standard deviation		2.38	0.13	
Standard error of measurement (based on the differences observed in the duplicates)		2.75	0.07	
WLO mice†				
1	8	88.66	2.31	2.61
2	8	83.36	2.41	2.89
3	8	83.69	1.84	2.21
4	8	86.13	2.05	2.38
5	8	88.92	2.29	2.57
6	8	88.19	2.26	2.55
Mean		86.49	2.19	2.54
Standard deviation		7.54	0.21	
Standard error of measurement (based on the differences observed in the duplicates)		6.04	0.13	
Mean weight CS mice		246 g (range 200-260 g)		
† Mean weight WLO mice		279 g (range 170-260 g)		

The results of the investigations performed on adult 12 week old male mice are on the other hand pointing towards a reduced production of corticosterone by the adrenal glands of CS males of this age.

First this assumption is supported by the results of the *in vitro* studies.

Second, the poorer response to corticotrophin measured by the repressed increase in plasma corticosterone levels after ACTH stimulation suggests hypofunctional adrenals in adult male mice. Since the peripheral plasma corticosteroid levels represent a balance between the rates of synthesis, release, utilization and degradation of the hormone, it is more difficult to interpret the results of plasma analyses.

TABLE 5

Corticosterone Production In vitro in Mouse Adrenals Incubated with ACTH. The Animals Were 4-Week-Old CS and WLO Males. Singly Housed and Resting. The Adrenals Were Removed and Bisected. After 1 re-incubation the Adrenal Halves Were Incubated with ACTH for 2 Hours and the Amount of Corticosterone in the Medium Was Measured. All Data Given Represent the Means of Duplicate Samples

Experiment No	No of mice	Corticosterone production $\mu\text{g}/100 \text{ mg adrenal tissue}/2 \text{ hours}$	Corticosterone production $\mu\text{g}/2 \text{ adrenals}/2 \text{ hours}$	Weight of 2 adrenals mg
CS mice				
1	8	96.88	1.24	1.28
2	8	91.49	1.18	1.29
Mean		94.19	1.21	1.29
Standard deviation		3.81	0.04	
Standard error of measurement (based on the differences observed in the duplicates)		3.01	0.29	
WLO mice†				
1	8	92.11	1.16	1.26
2	8	87.90	1.12	1.30
Mean		90.01	1.14	1.28
Standard deviation		2.82	0.03	
Standard error of measurement (based on the differences observed in the duplicates)		4.61	0.10	
Mean weight CS mice 125 g (range 90-145 g)				
† Mean weight WLO mice 116 g (range 80-130 g)				

However held together with the outcome of the incubations of adrenals with ACTH it seems justified to conclude that the results of the plasma analyses too are speaking in favour of an adrenal hypofunction.

Third the lower corticosterone content demonstrated in the adrenal glands of 12 week old CS males is consistent with a hypofunction or at least is a finding which is not contradictory to a decreased production of corticosterone by the adrenals. However no generalization is possible as to the relationship between the corticosteroid content and the secretory rate of the adrenal glands. Our findings give on the other hand additional support to the statement of Arnesen (1963) that the storage of steroids is reduced or abolished in permanently depleted adrenal glands.

The adrenal cortex of adult 12 week old male mice of the CS strain is known to be lipid depleted. The adrenocortical lipid depletion as such says nothing as to the functional state of the CS adrenal cortex. Loss of lipids of the adrenal cortex might as well be found after hypophysectomy as be the result of hyperfunction of the gland in the

latter case indicating a discharge of cortical lipids. Taken one by one none of the findings in the present study is necessarily indicative of adrenal hypofunction. However their association makes it likely that the adrenal gland of the adult male CS mouse is hypofunctional at least with respect to corticosterone production.

The last reservation is taken with an eye to the finding of Arnesen (1963) that the cortical cells in the lipid depleted adrenals of the adult CS mice were very rich in mitochondria. Arnesen stated that this finding was hardly compatible with the concept that the spontaneous lipid depletion reflected a state of permanent hypofunction of the cortex as postulated by Melcalf (1960). Arnesen stressed however that the term function in this connection was equivocal. One thing was the total or partial function in terms of specific hormone production, another was the basic and unspecific metabolic activity of the cells. The statement of Arnesen and the findings in our present study are therefore not necessarily conflicting. The possibility remains that the synthesis of corticosterone in the adrenal glands of the adult CS male in some way or other is reduced. In order to secrete the corticosteroids necessary to assure homeostasis the basic metabolic activity of the cortical cells might be enhanced and the cells present themselves as hyperactive in the ultrastructural picture as shown by Volbert & Arnesen (1960). More definite conclusions concerning the hypothesis here advanced are not yet possible.

SUMMARY

The adrenocortical function of male mice of the CS strain has been investigated. The prepubertal CS mice are known to have normal lipid pattern of the adrenal cortex whereas the elder adult CS mice have a spontaneous permanent lipid depletion of the adrenal cortex. The experiments included measurements of plasma corticosterone in ACTH stimulated animals, determinations of the corticosterone content of the adrenals in resting mice and measurement of the corticosterone production *in vitro* in adrenals incubated with ACTH. The inbred strain WLO with normal adrenocortical lipid pattern was used for comparative studies.

The study did not reveal any abnormalities in the adrenocortical function of prepubertal CS male mice. The result of the investigations on adult CS males 12 weeks of age were highly suggestive of reduced production of corticosterone by the adrenal cortex.

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CHROMOSOMAL ANALYSES OF ROUS SARCOMAS IN MICE—COMPARISON BETWEEN THE FINDINGS IN THE TUMOUR AND IN MATERIAL EXPLANTED IN VITRO

By

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In a previous investigation the chromosomal picture was analysed in 91 primary Rous sarcomas in mice (Mark 1967b). About 40 per cent of the tumours had a normal diploid stemline. Among the heteroploid sarcomas the stemline numbers ranged from 37 to 84. Hyperdiploid and tetraploid tumours 28 and 14 per cent respectively were the other two major components of the chromosomal spectrum while hypodiploid pseudodiploid and triploid sarcomas formed minor groups. These results were based on the findings obtained in fixations taken directly from the tumour (D). In fixations from tumour material explanted *in vitro* for a short period (Pc) 20 hours–7 days a deviating chromosomal picture was a common finding. A comparison between the findings in D and Pc from the same sarcoma was possible in the 81 tumours studied by both methods.

Among the 81 sarcomas 49 had a heteroploid stemline in D. In Pc only about $\frac{1}{4}$ of these heteroploid sarcomas showed the same or a similar stemline however and nearly $\frac{3}{4}$ had a normal diploid stemline. In this last group the stemline found in D had disappeared completely in approximately $\frac{1}{3}$ of the cases and in approximately $\frac{1}{2}$ it was refound as a sideline. Finally a small group about 8 per cent of the sarcomas with a heteroploid stemline in D showed a new heteroploid stemline in Pc. This *in vitro* normalization of the chromosomal picture naturally did not apply to primary sarcomas with normal diploid stemline in D. Many of them however had one or several heteroploid sidelines which were sometimes subjected to normalization *in vitro*. As in previous group there were only a few instances of new heteroploid stem sidelines in Pc.

This type of difference between D and Pc manifested as occurrence

TABLE 1
Stemline in vivo (D) and in vitro (Pe) in 27 Primary Tumours Subjected to Cross Tests

Tumour no	Sex	Stemline in H marker(s)	The stemline found in D is in Pe			Results of cross tests in table
			the same	stemline has normal diploid karyotype	not found stemline has normal diploid karyotype	
2	♀	37	-	-	+	3
3 sample I	♀	38	-	-	-	
5 sample I	♀	39	-	-	+	
12	♂	40	(+)	-	-	3
15	♂	40	(+)	-	-	
18	♂	40	(+)	-	-	
33 sample I	♂	40	(+)	-	-	
38 sample I	♀	40	(+)	-	-	
38 sample II	♀	40	(+)	-	-	
40	♀	40	(+)	-	-	
45	♂	40+	-	-	+	4
47	♀	40+	+	-	-	
49	♂	41	+	-	-	5
54	♀	41	-	-	+	
56	♀	41	-	+	-	
33 sample II	♂	41	-	-	+	
57 sample III	♂	42	-	-	+	
64	♀	42	-	-	+	
65	♂	42	-	-	+	
67	♂	42	-	-	+	
70	♂	42	+	-	+	
75	♂	42	-	+	-	
6	♂	57+	-	+	-	6
35 sample II	♀	57	-	-	+	
		67	-	-	+	
81	♂	74+	-	-	+	7
89	♂	80	-	+	-	

Only investigated in Pe but in op ration sample II IV the same picture *in vitro* as for tumour no. 2 and no.

of new heteroploid stem sidelines in Pc was regarded as an example of differential growth rate *in vitro* for tumour cells as discussed earlier (Mark 1967b). Concerning the other type of difference between D and Pc apparent as a normalization of the chromosomal picture in Pc arose the question of the real nature of the cells with a normal diploid karyotype. Two possibilities seemed to exist: (1) Outgrowth *in vitro* of normal stroma cells present in the cultured tumour fragments. (2) Overgrowth *in vitro* of a common second tumour stemline with a normal karyotype. The scope of the present investigation is to solve this problem. It might be performed by transplanting a tumour to a host with a sex opposite to the host for the primary tumour and then determining the sex *in vitro* of the outgrowing cells from explant of the transplanted tumour.

MATERIAL AND METHODS

The material consisted of 24 different primary Rous sarcomas in mice. The sex of the host and the designation for each primary tumour is given in Table 1. The numbering is the same as in previous investigation (Mark 1967b). In those cases where a primary tumour has been studied several times using specimens from subtotal operations that utilized for the present experiment is indicated by the sample number. Table 1 also shows the stemline numbers for each primary tumour and the occurrence of marker(s). The following columns illustrate the relationship between the findings in D and Pc of the primary tumours. Among primary sarcomas with heteroploid stemline the material includes three with similar or the same picture in D and Pc, four with a partial normalization of the chromosome picture *in vitro* and 12 with a complete normalization. The number in the last column refers to the table with the results for each tumour group.

The primary tumours were induced in mice of the inbred strain CBA by inoculation of a finely minced suspension of Rous chicken sarcoma strain Schmidt Ruppia (Mark 1967b). All animals used for transplantation were adult mice of the same strain. The primary tumours were transplanted during 1-11 passages. The new hosts were consistently selected to be of opposite sex to that of the mouse in which the sarcoma was induced (cross test).

The changes of the stemline of the primary tumour during transplantation were followed by direct fixations (D). The findings in direct fixation from the transplanted tumour served as a control of those from the primary culture (Pc). When a sarcoma was transplanted for several generations both D and Pc were performed intermittently.

All transplanted tumours were studied in Pc after 2-10 days of growth *in vitro*. In addition most sarcomas were investigated in Pc after a very short time of cultivation about 10-36 hours and after a comparatively long time *in vitro* 11-29 days. Often these P's were performed as a consecutive study of one or several passages.

The methods used for D and Pc were described earlier (Mark 1967a). All of the 199 determinations (Pc's) of stromal percentage have been made on at least 50 cells in each case except 10 in which the number of cells were on an average 33 and at least 25. The sex was determined karyotypically. Sexes were made by photographing some 4-5 cells in each tumour stemline and of material of each sideline.

As in previous reports stemline S is used to denote the most frequent karyotype in the populations of tumour cells and sideline (s) is used to denote other karyotypes occurring in a frequency of about 10 per cent or more of the population.

OBSERVATIONS

Diploid and hyperdiploid primary tumours were the two categories studied most extensively but all stemline groups which appeared in

the material of primary tumours were represented in the present investigation (Table 1) The results for the different tumour groups are recorded in Tables 2-7 which show the duration of the primary culture and the percentage of stroma cells appearing in each Pc The stemline among tumour cells *in vitro* and the stemline in D respectively are found in the two last columns

The cross tests i.e. transplantations of male or female tumours to hosts of opposite sex followed by sex determination of the cells in Pcs revealed the same fact in all cases Metaphases with a normal

TABLE 2
Cross Tested Tumours with a Hypodiploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	
2	I	36h	94	-	37
		5	98	-	
	II	20h	8	37	
		5	96	-	
	III	20h	0	37	37
		4	70	37	
		5	98	-	
		20h	10	37	
	IV	5	100	-	74
		20h	10	36	
	V	4	92	-	64+
		10	94	-	
	VII	20h	6	60-61+	60+
		24h	8	58+	
	VI	5	52	57+	57+
		7	78	57+	
		15	90	-	
3 sample 1	I	8	100	-	38
		4	78	38	
	II	5	96	-	
		18	58	38	
	IV	20h	0	74	74
		5	92	-	
	V	12h	14	76	
		24h	78	76	
		5	94	-	
		23	82	39	
	VII	20h	4	74	74
		6	80	38	
		14	62	72	
5 sample 1	I	20h	2	39	39
		4	92	-	
		12	88	(39)	

no designation for days

TABLE 3
Cross Tested Tumours with a Normal Diploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells and marker(s) = +	
19	I	24h	23	40	40
		5	80	40	
	III	11	80	40	40
	IV	3	33	40	
	V	4	48	40	
	VI	3	65	40	40
	VIII	10	94	-	
		24h	10	40+	40+
	IX	2	46	40+	
				40	
	IX	20h	8	40	
	X	6	44	39-40	
15	VII	20h	4	40	40
		4	56	78	78
	X	4	9	78	78
18	I	20h	25	40	40
		4	90	-	
		8	94	-	
	II	20h	14	40	40
		5	89	40	
		11	8	41	
	IV	20h	14	41	41
		2	68	41	
		4	89	41	
	V	5	40	41	41
		36h	78	41+	41+
		5	82	41	
	X	15	100	-	
		24h	49	41+	41+
		4	94	-	
III sample I	I	12h	2	41+	41+
		7	50	41+	
38 sample I	I	24h	48	41-49	41
		6	90	42	
III sample II	I	20h	0	43	43
		4	96	-	
		19	84	40+	
40	I	20h	4	43	43
		4	86	-	
		15	45	43	
II	I	20h	6	40	40
		5	70	40	
		18	30	±80+	
		20h	0	80+	9+

TABLE 3 (cont)

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells and marker(s) = +	
40	V	4	10	79+	77+
		11	10	79+	
		24h	11	77+	
	VIII	5	4	77+	77+
		20h	0	77+	
		7	2	77+	
	V	4	0	79+	79+
		10	2	80+	

TABLE 4
Cross Tested Tumours with a Pseudodiploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells and marker(s) = +	
45	I	1 ¹ / ₂ h	9	40+	40+
		5	54	40+	
		12	98	-	
	II	12h	4	40+	40+
		24h	10	40+	
		7	90	-	
	V	20h	11	40+	40+
		9	98	-	
		10h	0	41+	41+
	V	6	4	41+	
		13	29	41+	
46	I	4	75	40+	40+
		7	92	-	
	IV	20h	4	40+	40+
				80+	
	VI	4	18	40+	40+
		1 ¹ / ₂ h	0	40+	
		3	3	40+	

diploid karyotype had the same sex as the host. Thus they must represent stroma cells since this is the only component from the host present in the explants of the transplanted tumour. Among the cross tested sarcomas with a normal diploid stemline most of the outgrowing cells were stroma cells (Table 3) too. In all tumour groups however the degree of stromal growth was variable and above all influenced by the duration of the primary culture.

TABLE 5
Cross Tested Tumour with a Hyperdiploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells ■ and marker(s) = +	
49	I	3	9	41	41
		7	20	41	
		11	38	41	
	II	3	18	41	
		19	6	41	
	IV	20h	7	41	41
	VII	4	0	8?	
		4	6	32	8?
	X	19	■	43	
		2	28	40-41	40
		7	18	41	
		10	0	41	
54	I	20h	1?	41	41
		7	98	—	
		18	88	(4?)	
56	III	4	38	41	41
	VII	4	16	78	78
III sample II	I	7	8	42	42
		4	74	41	
		14	66	42	
57 sample III	I	4	20	42	4?
		13	20	42	
		4	98	—	
	III	7	96	—	
		17	92	42	
		36h	98	—	
	IV	8	70	41	42
		17h	8	81	
	V	6	94	—	81
		8	90	—	
		2	64	79	
	VI	29	0	42	79
		20h	28	81	
	VIII	4	95	—	81
		13	96	—	
		20h	1?	74	
64	I	3	88	—	81 74
		10	100	—	
64	I	20h	8	42	42
		7	94	—	
		11	96	—	
	II	4	26	41 4?	4?
65	I	20h	5	42	■
		4	96	—	
		8	9?	—	

TABLE 5 (cont.)

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) + +
		duration days hours	stroma cells per cent	tumour cells % and marker(s) + +	
65	II	5	98	-	
		12	98	-	
	IV	12h	16	43	43
		20h	26	43	
		5	96	-	
	VII	36h	98	-	43
67	I	3	2	43	42
	II	7	4	44	44
	IV	5	0	89	44
		7	22	44	
	V	3	4	87	87
	VI	3	48	85	85
	IX	3	20	49 84	49
		4	34	44	
	X	20h	2	42	42
70	I	20h	2	43	43
		6	96	-	
		10	98	-	

Before dealing with the details of this subject it might be convenient to survey the entire material in this respect. This is done in Table 8 and the mean values for the Pcs from all passages of all tumours are further plotted in the diagram in Fig. 1. In the entire material the percentage of stroma cells was lowest in fixations performed within 20 hours. During the following short period 21-36 hours the stromal values were increased to more than 50 per cent and then during the following 8 days there was a slow gradual increase to values around 70 per cent. After 10 days finally the total averages indicated a fall in the stromal percentage.

10-20 hours Already in the previous study of primary sarcomas (Mark 1967 a) it was found as a rule that fixations of Pcs performed before 24 hours after the explantation *in vitro* gave a result identical with or similar to the D. These observations were confirmed in the present study (Tables 2-8). The early Pc turned out to be an excellent method for chromosomal analysis of the Rous sarcomas and the results could be used for checking the findings in D. It is to be noted however that usually a minor fraction of stromal cells but occasionally a considerable fraction was found as early as 20 hours after cultivation (cf. 1 passage I of tumour 18 passage I of tumour 40 passage VIII of tumour 57 sample III and passage IV of tumour 65). A reduction of the duration of the primary culture to 10-12 hours usually caused

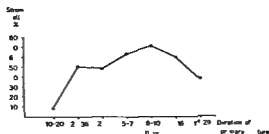


Fig 1

Mean percentage of stroma cells (in entire material 199 samples) after different periods of *in vitro* growth

an obvious decrease of the frequency of mitosing stromal cells in these cases (passage VI of tumour 18 passage V of tumour 57 sample III and passage IV of tumour 65). This indicates that normalization often proceeds rapidly. It seemed to be a rule that the percentage of stroma cells was lower during the first 20 hours than during the following 9-10 days but there were exceptions with similar stromal values or

TABLE 6
Cross Tested Tumours with a Triploid Stemline

Primary tumour no	Passage no	Primary cultures			Stemline in D S and marker(s) = +
		duration days hours	stroma cells per cent	tumour cells S and marker(s) = +	
36 sample II	I	4	95	-	62-63+
		2	92	-	
		3	94	-	
	IV	36h	65	62-63+	63+
	VII	5	100	-	
		12h	0	63+	
		8	98	-	
	X	20h	4	63+	
		3	58	62-63+	
		9	88	(61+)	
75 sample I	I	2	18	50+	50+
		10	66	51+	
		20h	0	50+	
	II	4	8	50+	49+
		16	0	50+	
		3	18	49-50+	
	VIII	4	14	49-50+	
		8	6	49+	
		17	10	50+	
76	I	20h	24	54+	54+
		5	98	-	
		3	100	-	

TABLE 7
Cross Tested Tumours with a Tetraploid Stemline

Primary tumour no	Passage no	Primary cultures		tumour cells % and marker(s)	Stemline in D S & 4 marker(s)
		duration days hours	stroma cells per cent		
51	I	4	30	74±	-1-
		7	5	74±	
		16	94	-	
	IV	20h	0	74±	-4-
		4	4	74±	
		5	2	73±	
	VII	15	4	73±	-4-
		2	6	73±	
		5	12	70-71±	
		12	50	74±	
99	VI	20h	9	70-75±	-4-
		6	60	40	

even a decrease in the frequency during the latter period, i.e. passages VIII and X of tumour 40 and passages IV and V of tumour 49, which means that the tumour cells are sometimes capable of outgrowing stroma from the beginning.

21 hours 11 days. The curves in Table 8 and the diagrams in Figure 1 obscure the fact that there existed at least three different patterns of stromal overgrowth during this period. The first and most common pattern was characterized by a very high degree of stromal overgrowth initially, often more than 90 per cent, and the high stromal content persisted during this period. Typical examples are found among the passages of tumour 51, sample III, tumour 65 and tumour 9, sample II. A second pattern was characterized by a stromal overgrowth in connection with the duration of the primary culture. Passage I of tumour 51 and passage I of tumour 49, for example, are typical of this pattern. The stromal overgrowth was pronounced and persisted in different degrees in passages I of tumour 49 and II of tumour 40, a low stromal content being observed in passages III and IV of tumour 49 and in passage III of tumour 40. The third pattern was characterized by a stromal overgrowth which appeared after the primary culture. This pattern was observed in passages I and II of tumour 49 and in passage I of tumour 40. The stromal overgrowth was pronounced and persisted in different degrees in passages I and II of tumour 49 and in passage I of tumour 40. The stromal overgrowth was pronounced and persisted in different degrees in passages I and II of tumour 49 and in passage I of tumour 40.

25 days. During this period two different patterns of stromal overgrowth were observed. In the first pattern, the stromal overgrowth was pronounced and persisted in different degrees in passages I and II of tumour 49 and in passage I of tumour 40.

Typical examples are passage I of tumour 54 passage I of tumour 5 sample I and passage VI of tumour 2. The other more common pattern during this late period was characterized by a more or less evident decrease of stroma cells. Examples are found among passages of tumour 67 sample III and passages of tumour 49. In these cases the decrease could be found in several passages but in other sarcomas it was only seen in one passage (for tumour 18).

Summarizing the findings for the whole period of *in vitro* growth 10 hours-29 days the following four patterns can be distinguished among the passages of the 24 primary sarcomas.

1 A continuous low fraction of stroma cells *in vitro* (example passages of tumours no. 49 and no. 76).

2 An initial low fraction of stroma cells gradually increasing and then usually decreasing in late Pcs (example passages of tumours no. 3 and no. 46).

3 An initial low fraction of stroma cells and a subsequent increase during the following periods but no definite correlation with the duration of the primary culture (example passages of tumour no. 12 and early passages of tumour no. 57 sample III).

4 An initial often considerable fraction of stroma cells rapidly increasing to a high level persisting for a long time and only some times decreasing in late Pcs (example passages of tumours no. 2 and no. 66).

With regard to the four patterns mentioned above there were often striking similarities between different passages of the same tumour and between these and the findings in the primary sarcomas (Table 1). It indicates that the patterns were related to fairly stable and specific characteristics of the individual sarcoma and were not due to random influences. However differences between passages of the same tumour were found in some cases. A few instances of an abrupt change in the *in vitro* pattern were recorded (passage I versus passage III of tumour no. 57 sample III passage I versus II of tumour 40 and passage V versus VI of tumour 67 are the most evident cases). In another category there was no real change in the *in vitro* pattern only a more or less apparent decrease in the stromal overgrowth after the first day *in vitro* (passages of tumour no. 46 and late passages of tumour no. 36 sample II and no. 45). In most instances the decrease was a parallel phenomenon to increase in passage numbers. It is to be noted however that this was a tendency found in some cases and it was not a detectable trend for the entire material (Table 8).

No distinguishable differences could be found between different number groups. Neither was it possible to correlate the decrease in stromal overgrowth with increasing passage numbers with any special karyotypic change. On the contrary the time patterns *in vitro* mentioned above often remained unchanged despite profound changes in the karyotype during transplantation. This constancy was shown by

TABLE 8
Mean Percentages of Stromal Cells in Primary

Period of primary culture	S number of primary tumour					
	< 2x	2x normal karyo type	2x pseudo diploid karyo type	2x normal and pseudo diploid karyo type	> 2x	± 3x ± 4x
10-20 hours	60(9)	88(11)	38(6)	71(17)	110(11)	70(4) 40(2)
21-36 hours	593(3)	345(6)	100(1)	310(7)	980(2)	620(1) -
2-4 days	830(4)	339(15)	320(3)	578(18)	374(21)	541(7) 170(2)
5-7 days	895(11)	524(10)	600(4)	546(14)	613(12)	785(4) 195(4)
8-10 days	940(1)	633(3)	980(1)	770(4)	747(6)	533(3) -
11-16 days	765(4)	545(6)	630(2)	566(8)	690(6)	00(1) 493(3)
17-29 days	820(1)	300(1)	-	300(1)	237(5)	550(7) 360(1)

Figures in brackets = number of samples in each group

for example tumour no 2 no 3 and no 18. In the first case tumour no 2 the stemline was probably doubled during passages and several markers appeared but still no definite change could be found in the *in vitro* pattern. However three cases with a probable correlation between karyotypic changes and changes in the *in vitro* pattern were found. They appeared among the few tumours with an abrupt alteration of the *in vitro* pattern. Thus in passage II of tumour no 40 there was a sudden decrease in stromal overgrowth at the same time as the stemline shifted to tetraploid level. After the shift to tetraploidy the stemline has undergone minor changes except for the appearance of double minutes (Vark 1967) and the picture *in vitro* has been stable. In passages of tumour no 49 and no 67 the hyperdiploid stemline was doubled but in late passages both were returned to the hyperdiploid region. In connection with this reversal there was a definite increase in stromal overgrowth in both cases suggestive of a correlation with the karyotypic change.

DISCUSSION

In the present investigation the same growth medium was used for all primary cultures *viz* Parker's tissue culture medium 199 supplemented with 20 per cent unfiltered inactivated serum from newborn calves. Another factor which may be regarded as constant is the genotype of the stroma cells which were all CBA. It is true that in different explants the stroma cells may vary in relative frequency and in their readiness for mitosis. However their competitive potential should be the same. Thus the cross tests actually constitute an estimate of the competitive capacity of the tumour cells versus the stroma cells. Under the existing conditions of the *in vitro* milieu the tumour cells

tures after Different Periods of *in vitro* Growth

In vivo passage no											Mean of entire material
I	II	III	IV	V	VI	VII	VIII	IX	X	XI	
5(11)	65(4)	00(2)	90(8)	93(3)	80(1)	47(3)	113(3)	80(1)	44(2)	10(2)	77(43)
2(3)	100(1)	—	815(2)	470(2)	—	850(7)	101(1)	—	240(2)	—	505(13)
1(14)	330(4)	III 7(6)	347(6)	260(7)	590(3)	475(4)	720(2)	270(7)	453(8)	30(1)	417(22)
2(12)	675(8)	997(3)	III 3(6)	580(4)	600(1)	655(4)	100(2)	440(1)	215(4)	640(2)	639(42)
0(4)	—	—	700(1)	940(7)	940(1)	940(1)	—	—	392(5)	—	707(14)
9(8)	570(4)	487(3)	—	—	—	553(3)	960(1)	—	390(2)	900(1)	600(27)
1(3)	60(1)	220(1)	—	870(1)	00(1)	180(2)	—	—	100(1)	—	374(10)

were in general inferior. With changed conditions however for example different media diverging results are not excluded. In the present study only one type of environment was tested.

The results obtained give a satisfactory explanation of the discrepancies observed between D and Pc. Moreover the results give an estimate of the magnitude of an important source of error in this type of experiment which has so far often been neglected. The common and often extensive stromal overgrowth *in vitro* was demonstrated for transplanted Rous sarcomas in mice. However a partial or complete normalization was found in many primary Rous sarcomas subjected to consecutive Pcs: there is no reason to believe that one was dealing with another factor in these cases.

The generally good correlation between D and early Pcs performed within 24 hours is probably not due to a real outgrowth of tumour cells. It seems more plausible that tumour cells ready for mitosis or even in mitosis will complete the mitotic cycle probably after a short lag period due to disturbances at the explantation. The critical period follows usually lasting for 10–14 days during which the stroma cells divide rapidly and outnumber the tumour cells. The artificial conditions *in vitro* with exclusions of normal mechanisms for control of growth seem to favour the normal stroma cells at the expense of the tumour cells. A shorter division cycle for the stroma cells is another factor which may influence the picture *in vitro*.

The growth *in vitro* of normal cells in general starts with a period of rapid divisions followed by a period of stagnation and often degeneration. The reappearance of a considerable number of tumour cells in old primary cultures is believed to be connected with this change in growth behavior of normal cells. The final picture during long term

cultivation was not studied. This would involve the question of transformation into permanent cell lines of either stroma cells, tumour cells or both.

Histologically it is not possible in most sarcomas to distinguish a stroma cell from a tumour cell. Judging from the results of the present and previous analyses the stromal content of most murine Rous sarcomas is probably very high. Rapid and extensive stromal overgrowth *in vitro* seemed to be especially common among sarcomas which were hard and fibrous and did not change their character during transplantation (typical examples are tumour no. 3 sample I, tumour no. 57 sample III and tumour no. 60). The differences between separate tumours were merely quantitative and the frequency of fibroblasts in an explant is probably of importance for the degree and rapidity of the normalization *in vitro*.

Most sarcomas became softer during transplantation and the time for outgrowth of the tumour was usually reduced to 10-14 days. A reduction of the stromal content paralleling the increased malignancy seems reasonable to assume. Both factors are believed to be responsible for the tendency towards reduced stromal outgrowth *in vitro* in late passages of some tumours.

Karyotypic changes of the stemline were not constantly connected with deviations in the *in vitro* pattern. However the chromosomal progression is still in the beginning in most sarcomas studied above and probably much of the original characteristics of the tumour cells are unaltered. Further a genotype successful *in vivo* is not necessarily successful in the *in vitro* milieu.

No significant differences could be found in the *in vitro* pattern between separate classes of stemlines. On the contrary each sarcoma seemed to follow its own pathway with regard to stromal overgrowth in primary cultures. This tendency was strengthened by the striking similarities in the *in vitro* pattern between different passages of the same tumour.

Despite the common use of *in vitro* methods for chromosomal analyses of tumours comparatively few investigations have been made of the relationship between D and Pc. Methods like the cross tests have been used too seldom and generally it has been impossible to exclude tumour stemlines with a normal karyotype and superior *in vitro* to the heteroploid stemlines. This controversial subject was discussed especially by Sandberg *et al.* (1961 and 1962) dealing with acute leukaemias in man. They found a significant increase of normal diploid cells as early as 10 hours after the initiation of the cultures and presumed that these normal elements represented nonmalignant cells. They point out quoting experiences by Bailly *et al.* and Ford that cultured leukaemic cells rarely grow as well as material from individuals without leukaemia and sometimes they do not grow at all. Baker & Atkin (1965) studying human lymphosarcomas and Hodgkin's

disease also found a high frequency of normal cells in their primary cultures and in some of these cases figures are given which show an increase of the normal cells with the duration of the primary cultures.

A similar situation seems to exist for bovine leucosis: heteroploid stemlines in D often disappeared in Pc and normal cells took over. Further there was occasionally a time pattern with a heteroploid stemline in the very early Pc and a normal finding in a later Pc (Basrur *et al* 1964; Hare *et al* 1964). Since stemlines with an apparently normal diploid karyotype have been found in D of both human and bovine leukaemia it cannot be concluded with certainty that the normal chromosome pictures in Pcs are due to overgrowth of normal cells but the suspicion is strong.

Stich (1963) referred to a case of a colonic adenocarcinoma in man having a hypotetraploid mode as judged from measurements of the DNA values but showing only normal diploid cells in Pc after 28 days *in vitro*. Spriggs *et al* (1962) found an altered chromosome picture in Ds from six cases of human carcinoma *in situ* of the cervix uteri. However, Richart & Corfman (1964) found a normal diploid stemline in five similar cases but only studied in Pcs. These are two additional examples where stromal overgrowth could be the explanation for the discrepancy between D and Pc and actually Stich expressed this opinion in his case.

There are also some related cases among animal tumours. Thus Hsu & Kellogg (1960) briefly referred to a chromosomal study of a primary culture of Novikoff's hepatoma of the rat where normal diploid cells suddenly dominated the picture in Pc. As stated this was certainly an example of stromal overgrowth *in vitro*. Further Hsu quoted by Basrur & Gilman (1963) observed that normal rat cells show a selective advantage over rat tumour cells in culture. The latter authors reported chromosomal studies of two strains derived from rhabdomyosarcomas of the rat. In one cell strain a heteroploid stemline persisted all the time *in vitro*. In the other strain there was a mixture of normal and heteroploid cells in the primary culture. However, about five months later the normal cells had outgrown the heteroploid stemline and this picture persisted during the reported observation time.

However, Nichols (1963) found no difference between D and Pc in the study of primary Rous sarcomas in the rat. This is of great interest since his tumours were induced by the same virus strain as the present tumours and further the primary cultures were grown in the same medium. Neither did Hellstrom *et al* (1962) find any differences between D and Pc in their investigations of primary and passed polyoma tumours in mice. They used similar methods for cultivation and Pcs were performed after intervals varying between 24 hours and 4 days.

Analyses similar to those performed in the present investigation will be necessary for interpreting the diverging results. For the time being

if only possible to assume that different tumours in different species may differ as regard their capacity of outgrowth *in vitro*

SUMMARY

The present study a comparison between the results obtained in direct fixations from the tumour (D) and in fixations from primary cultures (Pc) was prompted by certain findings in a previous study of primary Rous sarcomas in mice. In Pcs the original heteroploid stemline had often disappeared and been replaced by a normal diploid stemline. Several of the primary sarcomas were studied during transplantation and in Pcs the same phenomenon a normalization appeared. The present investigation showed that the discrepancy was due to stromal overgrowth *in vitro*. This process usually became manifest after 20 hours cultivation and as a rule the picture remained unchanged for 10-14 days. Subsequently tumour cells sometimes reappeared probably due to ceasing multiplication of stroma cells. The stromal outgrowth *in vitro* was most apparent in primary sarcomas and during early passages. In Pc from late passages *in vivo* the stromal overgrowth was sometimes reduced which might be due to a reduced stromal content and possibly to karyotype changes.

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APPLICATION OF ENZYME HISTOCHEMICAL METHODS IN THE DIFFERENTIAL DIAGNOSIS OF MELANOMA AND LEIOMYOMA OF THE IRIS

By

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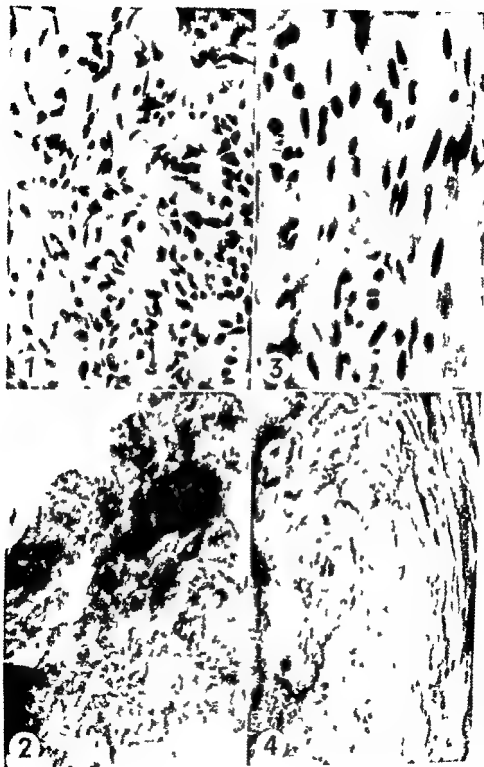
The difficulties in the histopathologic differential diagnosis between leiomyomas and malignant melanomas of the iris have been emphasized by *Rones & Zimmerman* (1958) and by *Hogan* (1964). There is no sharp clinical or pathologic distinction between these two groups in the majority of primary tumours of the iris. Usually gold impregnation Mallory's phosphotungstic acid hematoxylin stain and the thionin inclusion stain have been recommended for the differential diagnosis between the neurogenic and myogenic tumors of the iris (*Boke* 1956). Unfortunately these stains do not always produce sufficient information for the differentiation between melanoma and leiomyoma.

In a previous paper by us (*Niemi & Tarkkanen* 1964a) it was shown that the sphincter and dilator muscles of the iris have histochemical characteristics of muscle tissue by showing activity of phosphorilase but contrary to the smooth muscle tissue elsewhere in the body they also contain intense acetylcholinesterase activity. Furthermore two cases of leiomyoma of the iris (*Niemi & Tarkkanen* 1964b) showed strong acetylcholinesterase activity unlike leiomyomas of the skin (*Mustakallio et al* 1963). Melanomas of the iris have not been studied histochemically.

It is the purpose of this paper to point out the value of some enzyme histochemical methods in the histopathologic evaluation of melanomas and leiomyomas of the iris.

MATERIAL AND METHODS

The material consisted of five cases of iris tumours. The irises removed by iridectomy immediately after the enucleation of the eye were embedded either in mouse kidney tissue or in celloidin and frozen in a tissue holder. Sections were cut 10 to 40 μ in a cryostat, the preparations were placed on slides, let to dry for a couple of minutes and subsequently incubated for the demonstration of enzyme activity. Routine staining with hematoxylin in Mallory's phosphotungstic acid hematoxylin technique was performed on similar sections.



The methods for the demonstration of acetylcholinesterase (AChE), non specific holinesterase (ChE), monoamine oxidase (MAO) and phosphorylase activity were the same as employed in our previous study (Niemi & Tarkkanen 1964 a)

RESULTS

In two of the cases the tumor appeared both in hematoxylin eosin and Mallory's phosphotungstic acid hematoxylin stains as a leiomyoma (Fig 1 Cases 1 and 2 in Table 1). The two remaining tumors were very likely leiomyomas morphologically but phosphotungstic acid staining gave negative results (Fig 3).

With the aid of enzyme histochemical methods a good separation into two groups could be obtained in the entire series (Table 1). The first two leiomyomas showed high activity of specific cholinesterase (Fig 2) the activity of non specific cholinesterase being weak and localized to some coarse fibres in the tumor. A weak monoamine oxidase activity could be demonstrated in the tumor cells and also the amylo phosphorylase activity was clearly demonstrable. Since the pinkish colour of this reaction is difficult to demonstrate in black and white film no photomicrographs are available.

TABLE 1
Degree of Histochemically Demonstrable Enzymatic Activity in a Series of Four Tumors of the Iris

	AChE	ChE	MAO	Phosphorylase
Case 1	+++	(+)	+	+
Case 2	++	(+)	+	+
Case 3	—	—	+	—
Case 4	—	—	±	—

— no activity

(+) uneven slight activity

From + to +++ slight to intense activity

The remaining two tumors belong histologically to the melanoma leiomyoma group (Fig 3) but could not be classified to either of these types definitely. These tumors showed neither cholinesterase nor phosphorylase activity (Fig 4). A weak monoamine oxidase activity was present.

Figs 1-5

The Figures 1 and 2 refer to Case 1 and Figures 3 and 4 to Case 3, Table 1.

- Fig 1 Tumor of the iris as seen in hematoxylin eosin staining, frozen section $\times 800$
- Fig 2 The tumor shows intense AChE activity which appears to be located to the tumor cells $\times 480$
- Fig 3 Tumor of the iris composed of spindle cells with long slender nuclei and ill-defined cytoplasm. Hematoxylin eosin, frozen section $\times 800$
- Fig 4 AChE activity in this tumor appears negative. Compare the AChE activity with the intense activity seen in Fig 2 $\times 480$

DISCUSSION

Of the four iris tumors presented two were classified as typical leiomyomas with routine histological stains and the two others were considered as being probably leiomyomas although no myofibrils were detectable.

The muscular nature of the former tumors was revealed histochemically by the presence of phosphorylase activity. In terms of strong acetylcholinesterase activity, however, the tumors differed from other leiomyomas of the body (*Vustakallio et al* 1963) but accepting their origin from the sphincter or the dilator muscle of the iris the findings are in agreement with the results of the normal iris muscles (*Niemu & Tarkkanen* 1964a). The histochemical observations demonstrated on the other hand that the two other tumors did not exhibit characteristic features of an iris leiomyoma and belong therefore to melanomas. Recently a melanoma of the ciliary body managed through our procedure revealed similar results as these iris tumors.

The tumors of smooth muscle origin in the iris may show on the one hand a muscle pattern and on the other hand a neuroepithelial pattern and are therefore different from leiomyomas in other areas of the body as also suggested by *Reese* (1963).

A very interesting tumor of the iris of uncertain histogenesis has been described by *Dule & Dunn* (1958). No myofibrils were identified although the smooth muscle origin of the tumor was considered.

Rones & Zimmerman (1958) have emphasized that the tumors of the iris present a problem rarely encountered in those of the posterior uvea. They found it difficult to make a differential diagnosis between melanomas and leiomyomas of the iris although theoretically these tumors belong to different groups. They were able to divide their tumors of the iris into two groups. The first one included those with cytology characteristic of the nevus melanoma and leiomyoma types and the second group those capable of metastasis.

Apparently the application of enzyme histochemical methods may render more information of the nature of the tumors of the iris than what can be obtained by conventional staining methods. On the basis of our results at the Helsinki University Eye Hospital all tumors of the iris are routinely being frozen fresh immediately after the removal and processed through the enzyme histochemical procedure in addition to conventional staining methods.

SUMMARY

Four tumors of the iris removed by iridectomy and belonging to the melanoma leiomyoma group were frozen fresh immediately after the removal and stained by hematoxylin eosin, Masson's trichrome and Mallory's phosphotungstic acid hematoxylin stains. In addition acetylcholinesterase (AChE), non specific cholinesterase (ChE), monoamine

oxidase (MAO) and phosphorylase activity of the tumors were demonstrated by enzyme histochemical methods. Two of the tumors showed myogial fibres, an intense AChE activity, weak ChE as well as MAO activity but no clearly demonstrable phosphorylase activity. In the other two tumors no myogial fibres were demonstrable. There was no AChE or ChE nor phosphorylase activity but a weak MAO activity was present. It is concluded that application of enzyme histochemical methods will render more information about the nature of the tumors of the iris which very often present diagnostic problems rarely encountered in the tumors of the posterior uvea.

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THE MONONUCLEAR CELL INFILTRATE IN ALLERGIC CONTACT DERMATITIS

2 Selective Accumulation of Cells from the Bone Marrow

By

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Received 11 II 67

The characteristics of the mononuclear cell infiltrate in allergic reactions of the delayed type have been studied in several previous investigations most of which have employed transfusion of labelled cells between animals. In this way the immunological specificity of the infiltrating mononuclear cells has been studied with positive results (11-16) contrasting with negative ones (2, 6, 8, 10, 20). The source of the infiltrating cells has also been investigated using transfusion of labelled cells. Kosunen (10, 11) found that cells from lymph nodes regional to the area of sensitization with tuberculin accumulated in delayed hypersensitivity reactions in a higher degree than cells from non regional lymph nodes and spleen. Thymic cells were not found at all. However the effects of the handling of the cells during preparation of the cell suspensions, the significance of the host defense mechanisms (17) and the influence on the distribution pattern of the cells within the recipient caused by the rapid addition of a substantial number of cells to the circulation must be regarded as unsettled.

Attention has therefore been focused on experiments where the cells have been labelled and traced within the same animal. From those experiments the following picture of the cellular infiltrate in the delayed reactions of tuberculin and/or contact type can be deduced. The cells belong mainly to the lymphoid series (12, 21). Most of them are haematogenous, have recently proliferated and show a large proportion of macrophages and large lymphocytes (29). In contact allergic reactions cells labelled on the 4th day after sensitization have an increased mitotic activity (13). The mitotic activity of the infiltrating lymphoid cells in general does not seem to be correlated to the different stages of the development of the reactions as judged from the data of Kosunen *et al* (9) and Groth (7). Cells formed early in

mique as for H³-TdR. After the final injections these latter animals received intra peritoneally the same amount of H³-TdR (i.e. 20 μ c) as the animals labelled locally in the bone marrow.

In a preliminary experiment three animals labelled locally in the bone marrow were killed 24-48 hours after the injections for estimation of the efficiency of the bone marrow labelling and the degree of general labelling.

Testing

The testing was performed on day 10 at 8 a.m. i.e. $90 \pm 2\frac{1}{2}$ hours after labelling (21). The tests were applied on the hind part of the right midflank where the fur had been removed the day before by an electric clipper and by an electric shaver at least half an hour before testing. 20 μ l of a 0.90 per cent alcoholic solution of DNFB was applied with a micropipette to an area of 2 cm² (dose 20 μ g/cm², a dose shown to be atoxic in several tests on different guinea pigs during the last years).

Biopsies: Histologic and Autoradiographic Technique

48 hours after testing the material under study was collected. Skin biopsies were taken from the test sites and from untreated control skin taken cranially or caudally from the test site in every second animal of the two labelling groups.

The skin biopsies were fixed in carbonate buffered formalin, dehydrated in alcohols cleared in xylene and embedded in paraffin. The sections were cut with a microtome setting of 5 μ . Sections from both biopsies of each animal were placed in the same slides, their positions on the slides being changed alternately. A skin section from nonlabelled (cold) animals was also mounted on each slide.

For autoradiographs Kodak AR 10 tripping film was used. After an exposure time of 4 weeks the slides were developed and stained with haemalum.

The technical data given here are described in greater detail in a previous publication (13).

Evaluation of Autoradiograms

The criteria for the classification of cells as labelled were the same as those employed in a previous investigation in this series (13), i.e. lymphoid cells in corium with 3 grains or more were regarded as labelled. Counting of 2300 lymphoid cells in the corium from cold skin sections on 23 different slides from the experiment showed that 0.4 per cent of them had 3 grains or more.

The skin sections were first screened for determination of the types of lymphoid cells showing labelling and their location in the skin (corium or epidermis). A systematic counting was then performed by a specially skilled technical assistant unaware of the experimental design. From each of the two skin types of every animal 1000 lymphoid cells were counted in the upper parts of the corium, i.e. as a rule not more than one and a half high power view field (1000 \times) below the dermal-epidermal junction. These cells were counted from at least four sections from two different slides. The labelled cells with different grain counts were separated from each other in the counting protocol.

The lymphoid cells were identified as described by Roth (4) but macrophages (9) were also included in this group. The intensity of the allergic reaction was measured by the lymphoid cell response (4).

RESULTS

The results from the systematic countings of the autoradiograms are presented in Table 1 and 2. In Table 1 the values of the control biopsies have been subtracted from the values of the test biopsies in order to compensate for possible minor differences in labelling efficiency between the animals. Thus the difference between the values of test and control skin was used when comparing the animals. The test biopsies show a significantly larger increase in the proportion of labelled lymphoid cells in the group labelled locally in the bone marrow

than in the group labelled intraperitoneally ($p < 0.05$). The mean grain count values over the labelled lymphoid cells in test skin of the former group have increased whereas the corresponding values in the intraperitoneally labelled group have decreased (differences between the values significant $p < 0.01$). In Table 2 the absolute uncorrected figures for the mean grain counts and the maximal individual grain counts are given. The latter figures are very similar for both control and test skin from the intraperitoneally labelled animals and for control skin from the animals of the bone marrow labelled group. Only the figures for the test skin in the bone marrow labelled group are higher. However in 3 of the 8 animals the maximal individual grain count is below the highest of those in the intraperitoneally labelled group ($= 12$ grains). This is due to the fact that only 1 000 lymphoid cells were counted in the corium. This number represents but a small fraction of the cellular infiltrate and the more heavily labelled cells present may not be encountered in this counting. On screening of the autoradiograms however 12 grains was the highest number found in the intraperitoneally labelled group whereas cells with at least 30 grains were found in test skin sections from every bone marrow labelled animal. The lymphoid cell responses as a rule are low in this experiment but show a comparable distribution in the two animal groups. They show no correlations to the number of labelled cells as far as can be judged from this limited material.

TABLE 1

Autoradiographical values (Numbers of Labelled Cells and Mean Grain Counts) Have Been Calculated from Skin Sections of Two Groups of Guinea Pigs Sensitized to DNCB and Labelled with H^3 -TdR Locally in the Bone Marrow or Intraperitoneally

Test skin values minus control skin values			
Local bone marrow labelling		Intraperitoneal labelling	
No of labelled cells	Mean grain counts	No of labelled cells	Mean grain counts
13	18	7	-0.3
6	0.2	15	0.8
10	1.2	2	0.0
16	9.9	7	-1.5
10	-0.7	-3	-0.1
9	1.4	-1	-0.1
8	2.2	-4	-0.3
5	3		
Mean values	9.3	1.3	-0.2
St	1.0	0.4	0.3
Ranges	T 13-34	3 -6.5	9.36
	C 5-18	13.48	9.91

The differences between the values in test skin and those of untreated control skin are given in the table. 1 000 lymphoid cells have been counted in the corium. Each of the two skin types from every animal. C = control skin. T = test skin.

nique as for H³-TdR. After the final injections the latter animals received intra peritoneally the same amount of H³-TdR (i.e. 30 μ C) as the animals labelled locally in the bone marrow.

In a preliminary experiment three animals labelled locally in the bone marrow were killed 16-1 hour after the injections for estimation of the efficiency of the bone marrow labelling and the degree of general labelling.

Testing

The testing was performed on day III at 8 a.m. i.e. $20 \pm 2\frac{1}{2}$ hours after labelling (71). The tests were applied on the hind part of the right midfemur where the fur had been removed the day before by an electric clipper and by an electric shaver at least half an hour before testing. 20 μ l of a 0.90 per cent alcoholic solution of DNCH was applied with a micropipette to an area of 3 cm ($\approx 70 \mu$ l/cm² as it is shown to be toxic in several tests on different guinea pigs during the last years).

Biopsies: Histologic and Autoradiographic Technique

48 hours after testing the material under study was collected. Skin biopsies were taken from the test sites and from untreated control skin taken cranially or caudally from the test site in every second animal of the two labelling groups.

The skin biopsies were fixed in carbonate buffered formalin, dehydrated in alcohols cleared in xylene and embedded in paraffin. The sections were cut with a microtome setting of 5 μ . Sections from both biopsies of each animal were placed on the same slides, their positions on the slides being changed alternately. A skin section from nonlabelled (cold) animals was also mounted on each slide.

For autoradiography Kodak 4110 tripping film was used. After an exposure time of 4 weeks the slides were developed and stained with haemalum.

The technical data given here are described in greater detail in a previous publication (13).

Evaluation of Autoradiograms

The criteria for the classification of cells as labelled were the same as those employed in a previous investigation in this series (13): i.e. lymphoid cells in corium with 3 grains or more were regarded as labelled. Counting of 2 000 lymphoid cells in the corium from cold skin sections on 25 different slides from the experiment showed that 0.4 per cent of them had 3 grains or more.

The skin sections were first screened for determination of the types of lymphoid cells showing labelling and their location in the skin (corium or epidermis). A systematic counting was then performed by a specially skilled technician, assistant unaware of the experimental design. From each of the two skin types of every animal 1 000 lymphoid cells were counted in the upper parts of the corium, i.e. as a rule not more than one and a half high power view fields (1 000 \times) below the dermal-epidermal junction. The cells were counted from at least four slides from two different slides. The labelled cells with different grain counts are separated from each other in the counting protocol.

The lymphoid cells were identified as described by Roth (4) but in this study (9) were also included in this group. The intensity of the allergic reaction was measured by the lymphoid cell response (4).

RESULTS

The results from the systematic countings of the autoradiograms are presented in Table 1 and 2. In Table 1 the values of the control biopsies have been subtracted from the values of the test biopsies in order to compensate for possible minor differences in labelling efficiency between the animals. Thus the difference between the values of test and control skin was used when comparing the animals. The test biopsies show a significantly larger increase in the proportion of labelled lymphoid cells in the group labelled locally in the bone marrow.

general labelling is very slight. Intraperitoneal injection of the same amount of material is expected to give a more pronounced general labelling not favouring the bone marrow cells. If the bone marrow cells contribute to the mononuclear infiltrate at the test site, the test biopsies from the bone marrow labelled group should contain lymphoid cells with a maximal grain count exceeding that of the lymphoid cells in the intraperitoneally labelled group. If there is not only a random contribution but also a selective accumulation of bone marrow cells in the cellular infiltrate of the test site, the difference between the numbers of labelled lymphoid cells in the test skin and in the control skin should be larger in the group labelled locally in the bone marrow than in the intraperitoneally labelled group, provided the percentage of labelled lymphoid cells is comparable in the two groups.

Between $\frac{1}{2}$ –1 hour after local bone marrow labelling the cells in a control marrow in the same animal showed a grain count of 0 at the most. The mean grain count in this population is obviously very low. If cells from this population accumulate in the skin without mitoses after the labelling, however, part of them might be registered as labelled. This would make the mean grain count over the labelled lymphoid cells low despite the presence of some heavily labelled cells. If mitoses of these lymphoid cells not locally labelled occur between labelling and biopsy, they will not be detectable as labelled. Hence only cells coming from the locally labelled bone marrows will be registered as labelled, i.e. the mean grain count would be high compared with this value in the intraperitoneally labelled group. This argument would hold even if there was only a random accumulation of bone marrow cells. However, if this higher mean grain count occurs together with a percentage of labelled lymphoid cells at least as large in the group labelled locally in the bone marrow as in the intraperitoneally labelled group, it favours a selective accumulation of the bone marrow cells.

The results show that the differences between the numbers of labelled cells in test skin and in control skin are larger in the group labelled locally in the bone marrow than in the intraperitoneally labelled group. The differences between the mean grain count values are also more pronounced in the former group than in the latter at the same time as the proportion of labelled cells is similar in the two groups. According to the reasons given above, both these results favour a selective accumulation of bone marrow cells to the test site.

The increase in mean grain count values in the test biopsies from the bone marrow labelled group contrasts with the slight decrease of these values in the intraperitoneally labelled group. This latter decrease is in accordance with the results obtained in an earlier work (13) where it was made probable that the mitotic activity in some of the cells infiltrating the allergic reaction was elevated. This would give a dilution of the label. It is possible that this increased mitotic activity

■ present also in the locally labelled bone marrow cells. If so the initial grain counts are probably so high that in this group of animals the diluting effect of the mitoses is less pronounced than in the intra peritoneally labelled group.

The absence of labelled small lymphoid cells in this investigation is noteworthy. *Kosunen et al* (9) found an increasing proportion of labelled macrophages in the test lesions up to 48 hours the latest time chosen for biopsies in their experiment. These results were interpreted as indicating a transition of lymphocytes to macrophages. It is possible that such transitions (for ref. see 19) explain the absence of labelled small lymphocytes observed in the present experiment and the sparseness of such cells in the experiment of *McCluskey et al* (2) in which labelling was performed during the 36 hours preceding testing. In any case the available data do not permit any conclusions with regard to the question of which cell type is labelled initially and later accumulates in the test reactions.

The fact of heavily labelled lymphoid cells in the corium of control skin indicates that the lymphoid cells labelled in this experiment are not a normal constituent of the skin. They seem to accumulate only at the sites of inflammatory reactions. If this accumulation is immunologically specific answer or not is open to question. The results of *Blahovec, Sorkin & Turk* (1) may favour a special significance of the macrophages. These authors found that local passive transfer of cells from peritoneal exudate (which contained 25 per cent macrophages) resulted in delayed hypersensitivity to tuberculin as compared with transfusion of lymph node cells when the transfusion attempts failed. Cells of the macrophage type have been found to take up antigen in delayed reactions both in skin (3, 18) and in the regional lymph node (15). However the localization of the antigen was not influenced by sensitization to that antigen (3, 18). In both specific and non specific lesions cells labelled shortly before testing accumulated in the same degree (9). These latter findings indicate that at least the majority of these cells respond to a non immunological stimulus.

From the present experiment it is clear that cells from the bone marrow compose part of the mononuclear cell infiltrate in allergic contact dermatitis. About 3 days after labelling and 48 hours after application of the test they appear mostly as macrophages or large lymphocytes. Most of the labelled cells in the corresponding group injected intraperitoneally belong also to the same group of cells. This is true also for the cells labelled in the previously cited work of *McCluskey et al* (2) in both allergic contact dermatitis and delayed reactions of tuberculin type. These similarities justify the tentative conclusion that the large lymphoid cells in delayed hypersensitivity reactions in general are derived partly from the bone marrow.

SUMMARY

Two groups of guinea pigs sensitized to DNCB were labelled with tritiated thymidine either intraperitoneally or locally in the bone marrow. Autoradiographic analysis of sections from contact allergic reactions and from untreated control skin from each animal in the two groups was performed. Bone marrow cells labelled about 20 hours before epicutaneous testing were found to accumulate selectively in the 48 hour test reactions whereas no certain bone marrow migrants were found in control skin. Most of the labelled cells had the morphology of macrophages and large lymphocytes in both groups; thus no labelled small lymphocytes were found.

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CHOLERA FILTRATES AND AMMONIUM ION

Inhibitory Effect on Short Circuit Current of the Isolated Frog Skin

By

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Received 31 VIII 66

In 1960 it was shown by *Fuhrman* (4) that an inhibitor of the active sodium transport on the isolated amphibian skin was present in batches of a commercial preparation of cholera filtrate. The inhibitor was however not found in all batches of this filtrate (produced by Philips Duphar Holland). These observations have been confirmed (10).

Many attempts have been made to produce filtrates of *in vitro* cholera cultures with inhibitory effect primarily without success (5) but recently the production of active preparations have been reported (1). The present paper reports on the apparently successful production of filtrates capable of inhibiting the sodium pump.

During an analysis of the occurrence of the inhibitory factor in our filtrates there seemed to be a correlation between the pH of the filtrates and their inhibitory activity. The experiences reported by *Deplune & Mitchell* (11) that ammonia might have an inhibitory effect on the active sodium transport through the isolated frog skin therefore suggested a determination of the ammonia contents of the filtrates used for the frog skin experiments as well as experiments to determine the effect of ammonia on our frog skins.

The effect of vasopressin on the active sodium transport in the frog skin inhibited by cholera filtrates as described earlier (10) has been used throughout the present studies.

MATERIAL AND METHODS

Active filtrates have been produced mainly with the strain of *V. cholerae* designated 42, which has been used for the production of the commercial filtrate. Other strains have also been used as for example the fecal isolated strain of *V. cholerae* No. 51681. This strain and several other were obtained from the Pakistan SEATO

The study upon which this paper is based received financial support from the World Health Organization.

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Cholera Research Laboratory¹ Dacca East Pakistan Strain 51681 was isolated from a cholera patient on November 14th 1964. A strain of *V. coli* designated U 4 with the antigen formula O4 h3 H5 has also been used for some experiments.

The media used were peptone solutions in concentrations of 5 or 10 per cent Difco Bacto Peptone of several production numbers as well as Brunnengraber (Luback) peptone have been used. In some cases 0.5 per cent sodium chloride was added to the 5 per cent peptone but no effect of this addition was observed. The incubation time varied from 20 to 350 hours but was generally between 100 and 200 hours. The incubation took place in an air incubator at 37 °C.

The majority of the filtrates were made from cultures grown in 1 litre Erlenmeyer flasks containing 50 ml medium to give a high ratio of surface to volume (3). The media were inoculated with a loopful of bacterial culture from a solid medium. The cultures were centrifuged at 29 000 × g for 20 minutes after completion of the incubation and finally filtered by suction through membrane filter (Schleicher & Schull Nr 1121). Having passed sterility control the filtrates were distributed in small rubber stoppered vials and kept at -20 °C. Several recent batches of Cholera Filtrate Philips Duphar have been examined. The contents of the ampoules were dissolved in 10 ml distilled water.

When ammonia was used a 1.14 N NH₃ solution was adjusted with concentrated hydrochloric acid to pH 8.5. This solution was added to cholera filtrates, peptone solutions or directly to the solution bathing the outside of the skin in experiments made to investigate the effect of the ammonium ion on the sodium pump.

Filtrates of stools from cholera patients were obtained from the Pakistan SFA TO Cholera Research Laboratory Dacca East Pakistan. The stools had been sterilized by membrane filtration shortly after collection and were kept at +4 °C. Since arrival at this laboratory they have been kept at -20 °C. Details and bacteriological findings are given in Table 3.

The vasopressin used was Insipidin from A/S Alfred Benzon (Copenhagen) or Vasopressin synth Sandoz. The dose was 0.2 or 1 IU added to the solution bathing the inside of the skin.

The experiments were performed with the apparatus described by Ussing (13). The short circuit current was followed by frequent adjustment to zero of the potential over the skin.

The frogs used were mainly of the species *Rana temporaria* but also other non specified species as obtained in the sendings of frogs from southern Germany were used. In a few instances the abdominal skin from one frog was divided in halves and used in two parallel experiments. The experience was that this gave a very good control as the halves gave very similar results.

The cholera filtrates were added to the anatomical outside of the frog skin in amounts varying from about 0.5 ml to 10 ml. The same amounts of frog Ringer were added to the inside of the skin to make up for differences in hydrostatic pressure in the two compartments of the frog skin apparatus.

Measurements of the inhibition of the short circuit current of the isolated frog skin is not a method well suited for quantitative estimations. The variability is great and the measurements are slow, particularly because considerable time is required before the short circuit current stabilizes. In the present experiments it has generally been necessary to secure that the short-circuit current has been stable over a period of at least 20-30 minutes before the skin was found suitable for experimentation. This limited usually for the present purposes the working capacity of a frog skin apparatus to one or at the most two skins in one working day.

Estimation of pH was made electrometrically. Ammonium content was estimated by the microdiffusion method of Conway using the boric acid HCl method (14). This method will probably also measure volatile amines which might be present but the results are expressed as NH₃.

An attempt has been made to obtain a rough quantitative measure of the activities of different cholera filtrates by using a scoring system as follows:

¹ We are grateful to Dr Benenson and Dr Craig for their kind assistance in providing a number of freshly isolated cholera strains and filtrates of 1:1 from cholera patients.

We are grateful to Dr Hertberger Philips Duphar for supplying the batches of Cholera Filtrate.

Reaction	Description of variation in short-circuit current following addition of cholera filtrates	Figure
Total	Initially there might be a quick decrease immediately followed by an increase. The rise in current might continue over the initial level but is quickly followed by a steep decrease within 20 minutes after the application of the sample. No reaction to vasopressin.	1
Average	An initial current decrease immediately followed by an increase is sometimes observed. The increase may continue above the initial current level but is followed by a decrease of moderate steepness. Moderate positive reaction to vasopressin.	2
Doubtful	The initial current dive might be observed always followed by an increase in the current ascending considerably above the initial level. A subsequent slow decrease does not always reach the level of the current observed before the addition of filtrate. Positive reaction to vasopressin.	3
None	The initial dive in current might be observed followed either by no changes in current or a considerable increase. The current remains at the level achieved and reacts strongly to vasopressin.	4

We have not used the percentage decrease in current after application of the material to be tested because the reaction seems to be composed of two competitive reactions: one causing an increase, the other causing a decrease in the short-circuit current.

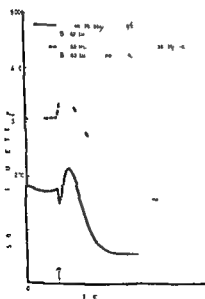


Fig. 1
Total reaction

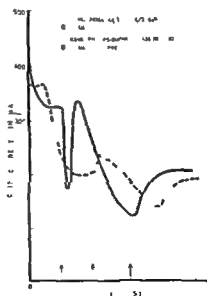


Fig. 2
Average reaction

TABLE 2
Cholera Filtrates Produced with 10 per cent Baclo Peptone

Filterate No	Incubation (hours)	Dose (ml)	Reaction	Organism			Nitrogen mg/100 culture vol	Date of ex
				Spec	Strain	pH		
110464/90	90	10	Total	V chol	47		31	21/4/64
-	-	5	Average	-	-		15	-
-	-	2.5	Average	-	-		8.5	-
-	-	1.25	Average	-	-		4.5	22/4/64
-	-	0.625	None	-	-		2.3	-
010664/170	120	5	Average	F coli	U4	8.6	20	23/4/64
-	-	2	Average	-	-	-	9.5	23/4/64
-	-	2	Average	-	-	-	-	11/8/64
-	-	2	Doubtful	-	-	-	-	13/8/64
-	-	1	Average	-	-	-	4.9	3/6/64
-	-	1	Average	-	-	-	-	4.6
-	-	1	Average	-	-	-	-	-
-	-	0.5	None	-	-	-	2.3	26/6/64
080664/120-U4	120	10	Total	-	-	8.61	40	9/8/64
-	-	5	Total	-	-	-	20	2/11/64
-	-	2	Average	-	-	-	8	10/6/64
-	-	2	Average	-	-	-	8	-
-	-	2	None	-	-	-	8	26/11/64
190365/163	168	5	Total	V chol	47	8.49	18.9	1/4/65
-	-	5	Average	-	-	-	-	8/4/65
-	-	5	Average	-	-	-	-	17/4/65
-	-	5	Total	-	-	-	-	13/4/65
-	-	5	Total	-	-	-	-	14/4/65
-	-	5	Total	-	-	-	-	20/4/65
140465/166	168	5	Total	-	-	8.38	15.5	23/4/65
-	-	5	Total	-	-	-	-	26/4/65
-	-	5	Doubtful	-	-	-	6.9	27/4/65
-	-	5	Doubtful	-	-	-	-	3/6/65
080465/314	314	5	Total	-	-	8.06	15	19/4/65
-	-	5	Average	-	-	-	-	2/4/65
010465-497075	168	5	Total	V chol	51681	8.63	20.1	11/5/65
-	-	2	Total	-	-	-	9.0	11/5/65
-	-	1	Doubtful	-	-	-	4.7	1/5/65
010465-471192	168	5	Total	-	-	8.41	21.3	17/5/65
-	-	2	Doubtful	-	-	-	9.5	18/5/65
110565-497075 Rug	168	5	Average	-	-	8.71	20	5/5/65
-	-	2	Total	-	-	-	9.2	26/5/65
-	-	2	Average	-	-	-	-	29/6/65
-	-	2	Total	-	-	-	-	30/6/65
-	-	2	Total	-	-	-	-	1/6/65
-	-	1	Doubtful	-	-	-	4.8	24/5/65
-	-	1	None	-	-	-	-	29/5/65
-	-	1	Doubtful	-	-	-	-	31/7/65

TABLE 2 (cont.)

Filtrate N	Incubation (hours)	Dose (ml)	Reaction	Organism			NH ₃ mM/l outside sol	Date of exp
				Spec	Strain	pH		
260363-472003	168	5	Total	-	-	8.63	18.3	3/6/63
-	-	2	Average	-	-	-	8.1	4/6/63
-	-	1	Average	-	-	-	4.2	8/6/63
-	-	0.5	Doubtful	-	-	-	2.2	9/6/63
-	-	0.2	Doubtful	-	-	-	2.2	10/6/63
260363-904659	168	1	Total	-	-	8.59	4.3	11/6/63
-	-	0.5	None	-	-	-	2.2	14/6/63
-	-	0.5	Doubtful	-	-	-	-	15/6/63
-	-	0.5	Doubtful	-	-	-	-	17/6/63
130663-472003	168	1	Average	-	-	8.57	4.2	24/6/63
-	-	1	Average	-	-	-	-	25/6/63
-	-	1	Average	-	-	-	-	26/6/63
190663-472003	168	1	Average	-	-	8.59	4.8	27/7/63
-	-	1	Average	-	-	-	-	27/7/63
-	-	1	Average	-	-	-	-	19/7/63
-	-	1	None	-	-	-	-	22/7/63
-	-	0.5	None	-	-	-	2.4	21/7/63
020763-904659	168	2	Average	-	-	8.63	8.7	12/7/63

TABLE 3
Stool Filtrates from Cholera Patients

Stool No	Dose ml	Reaction	pH	NH ₃ mM/l outside solution	Pathogen isolated
817	2	None	8.8	0.8	Inaba
1213	2	None	7.4	0.8	None
1214	2	None	8.2	2.1	None
1215	2	None	8.5	0.7	Inaba
890	2	None	-	0.4	Ogawa
917	2	None	-	0.8	Inaba
926	2	None	-	0.8	None
1016	2	Average	-	8.2	None

The first sending containing four stools (890 917 926 and 1016) was lost during transport for four months. The conditions under which the stool filtrates have been kept are not known.

seven with ammonia contents from 1 to 29 mM/l filtrate proved inactive when tested in 2 ml doses (0-2.1 mM/l outside solution). One filtrate contained 111 mM/l NH₃ and showed an Average reaction when tested in 2 ml doses (Table 3).

The addition of NH₃ was accomplished by using a NH₄Cl solution

containing 1.14 M/l NH_3 and adjusted to pH 8.5. The effects of different doses of this solution are listed in Table 3 and an example is shown in Figure 5. The correlation between the short circuit reaction and NH_3 content is in reasonably good agreement with the corresponding figures found in tests with the cholera filtrates.

TABLE 4
Philips Duphar Cholera Filtrate Batch P099

Date of experiment	Dose (ml)	NH_3 mV/l outside sol	Reaction
9/3/1964	10	4	Total
12/7/1965	5	5	Total
10/2/1965	4	1.5	Total
31/1/1964	2	0.8	None
31/1/1964	1	0.4	Doubtful

TABLE 5
Ammonium Chloride Solution 1.14 M/l pH = 8.5

Experiment No	Dose μ l	Reaction	NH_3 mV/l outside solution
1	100	Doubtful	4.6
2	150	Average	5.7
3	150	Average	6.8
4	150	Average	6.8
5	300	Average	13.7
6	400	Doubtful	18.2
7	400	Total	18.2
8	400	Total	18.2
9	400	Total	18.2
10	400	Total	18.2
11	400	Total	18.2

A few experiments have been performed with cholera filtrates where NH_3 was removed by the addition of sodium hydroxide. After storage for 24 hours at reduced pressure over diluted sulphuric acid (10 per cent) whereby the NH_3 was removed by evaporation, the pH was returned to 8.2 by means of concentrated hydrochloric acid. By diluting with sodium chloride free frog Ringer solution the isotonicity was restored and the final volume was adjusted to twice the original.

By this procedure the NH_3 content was reduced from about 120 mV/l filtrate to about 18 mV/l original filtrate. At the same time the inhibitory effect disappeared.

Addition of NH_3 solution to obtain the original concentration of NH_3 restored the inhibitory effect.

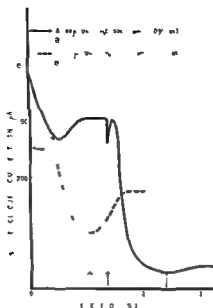


Fig 5

The effect of NH_3 on the isolated frog skin

DISCUSSION

The experimental demonstration of sodium pump inhibitors in Cholera Filtrate Philips Duphar (4) and in stools from cholera patients (6) supported the theory that the diarrhoea in cholera was caused by an inhibition of active absorption of sodium from intestinal lumen to blood.

The inhibitory activity was however not always found in these preparations and many attempts to produce the inhibitor by growing *V. cholerae* in different media were unsuccessful (5).

We have prepared active filtrates with 5 and 10 per cent peptone solutions using prolonged incubation. With such preparations typical responses were regularly obtained using doses between 1 and 10 ml.

A series of control experiments with uninoculated 5 per cent and 10 per cent peptone solutions have consistently shown only a rise in the short circuit current and no subsequent decrease.

Attempts to develop a quantitative method for estimating contents of inhibitor were unsuccessful due to the variability of the test system. Therefore the scoring system described was adopted. The isolation or concentration of the inhibitor failed. Even the very gentle gel filtration method was not successful. The inhibitory effect disappeared after passage through a column of Sephadex G 20 and it was not possible to elute any inhibitory principle from the columns by various eluents.

Considerable difficulty was encountered due to changing sensitivity of the frog skins from winter to summer. It seems to be that the sodium pump is almost unsusceptible to certain agents during winter but that during spring the sensitivity is rising simultaneously with the start of the mating activity.

When tested in the spring and summer seasons filtrates produced on 5 or 10 per cent peptone media proved active in inhibiting the short circuit current. Roughly the activity was doubled by increasing the peptone concentration from 5 to 10 per cent. The doses necessary to cause average reaction were 3.5 and 2.0 ml respectively. For comparison the dose employed of the originally used commercial filtrates (10) was only 0.5 ml.

During the Cholera Symposium held in Honolulu in January 1967 it was described (11) that ammonium ions in a concentration of about 4 mM/l in the solution bathing the outside of the frog skin had an inhibitory effect on sodium transport as measured by the short circuit current.

A similar effect has been reported earlier by Huf (7, 8) who presented data showing the inhibitory effect of NH_4^+ ions at $\text{pH} \approx 7.5$ when substituting K^+ ions in the solution bathing the outside of the skin.

It appears that the influence of ammonia on the active sodium transport when applied in ordinary frog Ringer solution depends on the pH of the solution so that inhibition is observed regularly only at pH higher than 8 (9).

We examined the NH_3 content of many of our cholera filtrates using the method of Conway, as already described, and as may be seen from the Tables 1 and 2 a good correlation was found between the contents of ammonia or ammonia like substances in cholera filtrates and their inhibitory effect on the active sodium transport.

It was also shown that NH_4^+ ions (or NH_3 molecules) in the form of a solution of ammonia in hydrochloric acid with a pH of 8.5 would cause an inhibition of the short circuit current very similar to the effect of cholera filtrates.

Human cholera stools with low NH_3 content did not inhibit the short circuit current whereas such inhibition was observed with a single stool filtrate with higher NH_3 content.

With a recent particularly active Philips Duphar cholera filtrate inhibition was observed after applying 4 ml filtrate which is equal to 1 mM NH_3 per litre outside solution. The ammonium content is considerably lower than that found in filtrates of our own preparation. However the amounts of filtrate necessary to yield a total response were higher than the dose used with the filtrates tested earlier (10).

The question about the identity of the inhibitor found in our filtrates (which we believe is ammonia) and the inhibitor found in the Philips Duphar filtrates previously tested cannot be finally settled as nothing is left of these old filtrates.

Certain arguments may be formulated against the identity. For example the incubation time used for the production of the commercial filtrate is only 16 hours on semi solid media which in our experience is too short a time for the accumulation of sufficient amounts of ammonia in liquid media. Our observation with a recent Philips Duphar filtrate mentioned above where inhibition of the short circuit current was obtained with amounts of filtrate yielding as little as 1.5 mM ammonia per litre outside solution seems also to indicate that the inhibitor in this filtrate is of another kind than that found in ours. Total inhibiting doses of our filtrates generally created an ammonia concentration from 10 to 20 mM NH_3 per litre outside solution in agreement with the concentration needed of a pure ammonium chloride to effect similar reactions on the frog skin. The observation by Fuhrman (6) that the inhibitory activity resisted heating with alkali seems to exclude that the inhibitor in the Philips Duphar filtrates could be ammonia.

On the other hand the reactions observed on the short circuited frog skin are very similar in all details. The inhibitor is in both preparations able to pass a dialysis bag and it has in our hands been impossible to purify the inhibitor from either source by means of gel filtration.

This in connection with the low specificity of the test system suggests that the inhibition found in our cholera filtrates and perhaps also in stools from human cholera patients at least partly is caused by contents of ammonia or ammonia like substances.

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PRECIPITINOGENS IN BETA HEMOLYTIC STREPTOCOCCI
AND SOME RELATED HUMAN KIDNEY ANTIGENS

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Received 3 x 66

The recognition of acute glomerulonephritis as a sequela to a recent infection with nephritogenic streptococci has been well established by means of epidemiological studies (Rammelkamp *et al* 1952, Rammelkamp 1953 and Wertheim *et al* 1953). The nephropathogenetic role of the streptococcus is however still a matter of controversy. Besides a direct toxic or enzymatic action of streptococcal products such as streptolysin S (Tan & Kaplan 1962) or DNase (Bernheimer *et al* 1957) various immunological explanations have been discussed (reviewed by Cruickshank 1963, Dixon and Feldman 1964 and Cluff & Johnsson 1965). Gamma globulin and complement (Vellors & Ortega 1956, Freedman *et al* 1960, Freedman & Markowitz 1962 and Lachmann *et al* 1962) as well as streptococcal antigens (Seegal *et al* 1959) and antigen antibody complexes (Andres *et al* 1966) have been demonstrated in glomeruli from patients with acute glomerulonephritis. These findings may partially elucidate the immunopathogenetic mechanism of streptococcal induced glomerulonephritis.

The antigenic composition of cell walls from beta hemolytic streptococci belonging to Lancefield's group A have been extensively examined (e.g. Lancefield 1954, Krause 1958, Krause and McCarty 1961, McCarty 1964) and the biological significance of these antigens has also been investigated (Lancefield 1954, McCarty 1964, Holton & Schwab 1966, Abdullah & Schwab 1966). Although the streptococcal antigens in culture filtrates (reviewed by Halbert 1964) have been intensively studied few reports are available concerning the intracellular streptococcal antigens (Wilson & Wiley 1963, Wahl *et al* 1965). That such studies might be of special importance in relation to the immunopathogenicity of streptococci in acute nephritis was illustrated by Markowitz & Lange (1964) and Kaplan (1964, 1965) who could show antigenic relationships between human tissues and streptococcal cells.

The aim of the present investigation was to investigate the intra

cellular antigenic composition of streptococcal cells belonging to Lancefield groups A, C and G and to compare these antigens with those occurring in ordinary Lancefield extracts of cell walls as well as to antigenic factors derived from normal human kidney.

MATERIAL AND METHODS

Bacterial strains. Fourteen strains of beta hemolytic streptococci belonging to Lancefield's groups A, C and G were investigated. These strains are listed in Table 1. Primary human pathogenic strains are designated with type numbers. The reference strain 13846 (type 12 M+) was isolated from a patient with an acute glomerular nephritis in Bedale, England 1963. This strain and the non nephritogenic type 12 streptococcus (strain 8300) were obtained from the Central Public Health Laboratory, Colindale, London. The other streptococcal strains were kindly supplied by Dr. Rebecca Lancefield, The Rockefeller Institute, New York. In addition two pneumococcal strains (types 27 and 2 R) obtained from Statens Serum Institut, Copenhagen, were employed.

Antigen Preparations

Streptococcal intracellular material (IC). Transfers from 16 hours glycerine broth stock cultures of the streptococcal strains were grown in an antigen free medium under stabilized pH conditions at pH 6.8 as described by Holm & Falsen (1966). After 16 hours cultivation the cultures were centrifuged at 1500 $\times g$ for 30 min at 4°C. The bacterial cells were suspended in 10 volumes of buffered saline (0.15 phosphate buffer pH 7.0), centrifuged and washed three times in the same way. The final bacterial sediment was suspended in 3 volumes of buffered saline sonicated in a Raytheon 200 watt 10 kc oscillator for one hour under chilling with cold tap water (<10°C) and subsequently freeze pressed five times in the x press described by Fidebo (1960). After thawing the material was mixed with glass beads (7 mm) and shaken at 4°C for three hours, decanted and centrifuged at 12000 $\times g$ for 30 min. The supernates were designated IC (e.g. IC A1² (P 13846), IC A3 (S 84) etc.). The protein content of the IC preparations varied between 4.1 and 6.0 mg/ml as measured by the method described by Lowry *et al.* (1951) using lyssozyme as a reference substance. Three to five IC preparations from each strain were analyzed and the one with the most complete precipitinogenic composition was employed in the investigation.

Lancefield extracts (CM). The bacterial sediments obtained after the preparation of the IC material were washed three times with distilled water and centrifuged at 12000 $\times g$ for 20 min. These sediments were used in the preparation of Lancefield extracts (boiling at pH 2.0 for 10 min., followed by neutralization and centrifugation). The preparations were designated CM (e.g. CM S 84, CM P 13846).

Streptococcal polyglycerophosphate antigen. This preparation was obtained as a gift from Dr. Maclyn McCarty for preparative procedure see his report (1959).

Human kidney antigen (K sup & sed). The source of these materials was the kidneys of a six week old boy who died of heart failure. They were removed immediately after death, washed with saline, chilled in an ice bath and homogenized with 3 volumes of buffered saline in an Ultra Turrax at 14000 rpm for three minutes under chilling. After centrifugation at 3000 $\times g$ for 30 min. a kidney tissue supernate (K sup) containing 3 mg protein/ml and a kidney tissue sediment were obtained. This sediment was suspended in an equal volume of buffered saline (pH 7.4) and was designated K sed. Both preparations were bacteriologically sterile.

Human lung antigen was prepared from lung tissue of the same origin as the aforementioned kidneys using the procedure just described. No streptococcal contamination was noted in this preparation.

Pneumococcal intracellular antigen was prepared in the manner earlier described for streptococcal intracellular material.

Antisera

Anti-streptococcal serum (anti IC + FC) was produced in a sheep which had been immunized by repeated subcutaneous injections of IC material and culture filtrates.

(IC) from the eight group A streptococcal strains using an adjuvant technique. Each injection consisted of 0.5 ml pooled IC + 10 ml pooled EC. The mixture was emulsified with an equal volume of paraffin oil Arlacel® (9 vol:1 vol). The immunization schedule implied weekly injections for 8 weeks followed by monthly injections for one year. The antiserum bleeding employed in the present investigation was taken one week after the twelfth injection and was designated Anti IC+EC. No antibodies could be detected in anti IC + EC against the medium used for cultivation of the streptococci. Portions of this antiserum were absorbed with heated or unheated IC materials from different streptococcal strains. The absorptions were carried out by adding an aliquot of the absorbing antigen to a portion of the antiserum incubating at 37°C for 1 hour and storing at 4°C overnight. The mixture was centrifuged at 10,000 ×g at 4°C for 30 min. and the supernate decanted. If precipitins to the absorbing antigen were still detectable the procedure was repeated.

Anti human kidney serum (anti K | P) Four rabbits were injected subcutaneous 1/2 once a week for 8 weeks with an emulsion prepared by mixing 0.5 ml kidney with 0.5 ml paraffin oil - Arlacel A® (9 vol:1 vol). A booster dose of 0.5 ml kidney in 0.5 ml saline was given intravenously three weeks after the eighth injection and bleedings were performed one week later. The sera after separation from the blood cells were repeatedly absorbed as described before with freeze dried pooled human plasma lacking demonstrable streptococcal precipitins. The absorbed antisera were tested by the double diffusion in gel method and found free from antibodies against serum proteins in addition to antibodies against constituents of the streptococcal medium could be detected. The serum which contained the largest number of precipitins when tested with kidney was chosen and was designated anti K | P.

In some experiments the anti K | P absorbed with human Al(+) erythrocytes was used. This absorption was performed by suspending a sediment of erythrocytes (1 volume) in anti K | P (2 volumes). This mixture was incubated for 1 hour at 37°C followed by centrifugation at 1,000 ×g for 10 min.

DNase inhibition test Analyses for the detection of DNase inhibiting capacity of IC materials from different streptococcal strains were made according to a technique recently described by Holm & Kayser (1966).

Diffusion in gel analyses The double diffusion in gel method of Ouchterlony (1967) was employed in the gel chamber modifications described by Wadsworth (1967) and Holm (1965). The interpretation of the comparative precipitation patterns was made according to the principles described by Ouchterlony (1962).

Immunoelectrophoresis Immunoelectrophoresis and comparative immunoelectrophoresis were performed according to the techniques described by Wadsworth & Hansen (1960). To obtain optimal separation of the streptococcal antigens the antigen reservoir had to be placed 4 cm from the cathode and the separation was made on 9 × 12 glass plates applying a field strength of 7 V per cm for 80 min.

Registration of precipitates Photographic records of the precipitation patterns were made by means of Polaroid panchromatic film reproductions according to Wadsworth (1963).

EXPERIMENTS AND RESULTS

Reference Streptococcal Precipitation Spectrum

To facilitate comparison of the various antigenic materials a reference streptococcal immune system was established. The reference antigen consisted of the IC A12 (P 1384b) preparation and the reference antiserum was the polyvalent sheep anti IC + EC. Fig 1 a is a photograph of the spectrum developed by the undiluted reference antigen and the reference serum. Additional precipitation lines were observed when one of the reactants was diluted. The reference precipitation spectrum formed by these reactants consisted of eighteen precipitation bands. Fig 1 b. By dilution of the reference antigen or the reference serum it could be verified that these bands represented separate immunological systems. The lines were designated aA - iT.

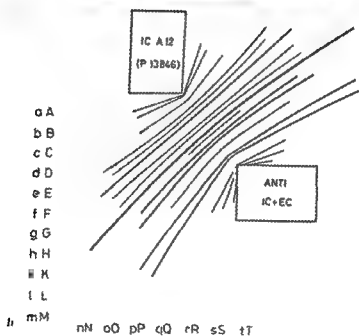


Fig. 1

The reference streptococcal immune spectrum

- a) Photograph of the precipitation pattern obtained with undiluted reference reactants: Upper basin: reference antigen IC A12 (P 13846); lower basin: reference immune serum anti IC+EC.
- b) Schematic drawing of the separate immune systems comprising the total reference spectrum

labelling from the antigen basin. The antigenic factors are represented by small letters and the corresponding antibodies by capitals. The most dense and rapidly appearing line in the precipitation pattern was the $\alpha\alpha$ line while the $\alpha 1 - \alpha E$ lines were fainter, less distinct and appeared late in the diffusion analyses.

The influence of heat on the precipitinogens in the reference antigen was examined by testing samples after heating them at 60, 80, 100 or 120 °C for 30 min. The heated preparations were tested with the comparative double diffusion in gel technique employing the reference streptococcal immune system. The results of these analyses are shown in Table 1. As may be seen the c , g and k factors could not be detected after heating the reference antigen to 60 °C for 30 min while the factors n , p and q were still demonstrable after heating to 120 °C for the same period of time.

The reference antigen was also treated with trypsin (1 mg/ml Difco 1:200) for 60 min at 37 °C, pH 8.0; the enzyme action was blocked by addition of trypsin soy bean inhibitor (NBC 1 mg/ml). It may be seen in Table 1 that the factors c – f , h , i , n , o , q and r were not destroyed by the trypsin treatment and n and q resisted heating at 120 °C for 30 min followed by exposure to trypsin.

Immunological Comparison of the Different IC Preparations with the Streptococcal Reference Antigen

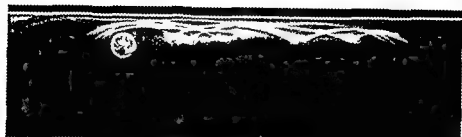
The streptococcal IC preparations were analyzed with the established reference immune system by means of the comparative diffusion in gel plate technique. The results of these analyses are given in Table 2. The relative concentration of the identified factors is not indicated in the table. As some of the lines in the reference spectrum were faint and did not extend far into the area of interference, the coalescence of these lines with those formed by the reference immune serum and the IC preparation was sometimes uncertain. Such reactions are indicated by question marks. It may be seen in the table that streptococcal strains which are considered as primary human pathogenic, i.e. the group A strains, types 7 and 20 in group C and the T 16 strain of group G had the a , b , g and k factors. These factors could not be detected in the other three strains tested. Factor f was only found in group A streptococci. Factors n and q were present in all the A, C and G streptococcal strains and also in the two pneumococcal strains tested (pneumococcus types 27 and 2 R). Factors c and i were detected with certainty only in the antigen preparation from the reference strain and factor p only in the type 3 and the type 16 strain in addition to the reference strain. The factors r and s were found in all the group A, C and G streptococcal strains tested. Similar results were obtained concerning the s and t factors although some uncertainty existed with regard to strains T 20 and K 39. Factor d was found in all group A and G strains but only with certainty

TABLE I
Resistance of *Yersinia* in the Reference Antigens to Heat and Trypsin

	a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	r	s	t
Reference antigen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Untreated control	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+	+
60 C. 30 min	-	-	-	-	-	-	-	+	-	-	(+)	+	-	+	+	+	-	-
80 C. 30 min	-	-	-	-	-	-	-	+	-	-	-	+	-	+	+	-	-	-
100 C. 30 min	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	-	-
120 C. 30 min	-	-	-	-	+	+	-	+	-	+	-	+	+	-	+	-	-	-
Trypsin 90 min	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-
170 C. 30 min + trypsin 60 min	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-

TABLE 2
*Precipitogens of the Reference Streptococcal Antigen Identified in Intracellular
 Materials from Streptococcal Strains Belonging to Different Types and Groups*

Beta hemolytic streptococcus			Total number of precipitino genic factors	Identified precipitinogenic factors																			
Group	Type	Strain		a	b	c	d	e	f	g	h	k	l	m	n	o	p	q	r	s	t		
A	12	I 13846 Reference strain	18																				
	1	T 1-119 7	16	+	+	-	+	+	+	+	+	+	-	-	+	-	-	+	+	+	+		
	3	S 84	16	+	+	-	+	+	+	+	+	+	?	+	+	+	+	+	+	+	+		
	4	T 4 95-11b5	17	+	+	-	+	+	+	+	+	+	-	+	+	-	?	+	+	+	+		
	19	8300	17	+	+	-	+	+	+	+	+	+	-	+	+	?	-	+	+	+	+		
	25	B 346 op	16	+	+	-	+	+	+	+	+	+	?	+	+	?	-	+	+	+	+		
	28	F 98 150A-4	18	+	+	-	+	+	+	+	+	+	?	+	+	?	-	+	+	+	+		
	10	B 737 34 31	17	+	+	-	+	+	+	+	+	+	?	+	+	+	-	+	+	+	+		
	C	7	T 7 J	14	+	+	-	-	+	-	+	+	+	-	-	+	?	?	+	+	+	+	
		20	T 0 (Nell)	15	+	+	-	+	+	-	+	+	+	?	+	+	-	-	+	?	+	+	
		S P A K 39	9	-	-	-	?	+	-	-	+	-	-	+	+	-	-	+	?	?	?		
		K 64 0-13	11	-	-	-	+	+	-	-	+	-	-	?	+	-	-	+	+	+	+		
C	16	T 16 J	16	+	+	-	+	+	-	+	+	+	?	+	+	+	+	+	+	+	+		
		F 68 A	10	-	-	-	+	+	-	-	-	-	-	+	+	-	-	+	+	+	+		
Not primary human pathogenic streptococcal strains																							



a

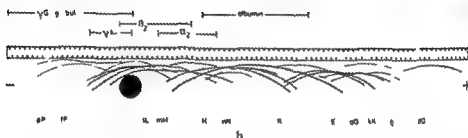


Fig. 2

Photograph (a) and schematic drawing (b) of the immunoelectrophoretic spectrum obtained with the reference streptococcal reactants. The reference antigen IC 4¹⁹ (I 1384b) was applied in the circular basin and electroseparated. The reference immune serum anti IC+IC was placed in the longitudinal basin. The precipitate designations correlate with Fig. 1.

in two of the four group C strains. The factor *h* was present in all A and C strains and the type 16 strain but could not be demonstrated in the other group G strain. The analyses for the detection of the *o* factor were uncertain due to the faint precipitate formed by this antigen and its antibody. The *m* factor was found in all group A strains with the exception of the type 1 and all group G strains but only in one of the four group C strains investigated. All IC materials from strains showing the presence of the *m* factor had DPXase inhibiting capacity. No antibodies were found in the anti IC+IC serum corresponding to the group A specific carbohydrate in the I media extracts or the polyglycerol phosphate preparation.

Immunoelectrophoretic Analysis of the Reference Streptococcal Antigen

In order to further characterize the precipitinogens in the reference streptococcal antigen the reference antigen was separated electrophoretically in a gel and analyzed with the reference immune serum. Fig. 2 a and b present the immunoelectrophoretic precipitation pattern which consisted of at least 19 precipitation arcs most of which were localized on the anodal side of the antigen reservoir. Many of the reference precipitinogens were found on the anodal side of human serum

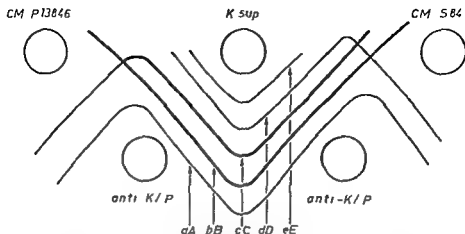


Fig 3

Schematic drawing of the precipitation pattern obtained when Lancefield extracts from a nephritogenic (CM - P 13846) and non nephritogenic (CM - 584) streptococcal strain were analyzed with the kidney anti kidney immune system. Precipitate designation is indicated.

albumin Bv means of the comparative immune electrophoretic technique and employing heated and trypsinized preparations of IC materials from different strains as well as absorbed antisera twelve of the precipitation arcs could be identified with the precipitation bands in the aforementioned established reference precipitation spectrum. These immune systems are labelled in Fig 2 b. The identification of the other immune systems was not possible to perform due to the faintness of the precipitates or the multiplicity of precipitation arcs in certain regions. Immune electrophoretic analyses performed at lower pH values (down to 5.8) than the one used in the standard technique did not permit further identification.

Immunological Relationship between Streptococcal Cells and Human Kidney

Double diffusion in gel analyses of human kidney antigen (K sup) with anti human kidney serum absorbed with human plasma (designated anti K/P) revealed at least five separate immunoprecipitates. These were designated aA - eE (Fig 3). When Lancefield extracts of the nephritogenic reference streptococcus (CM - P 13846) and a non-nephritogenic streptococcus (CM - 584) were analyzed with the same technique against anti K/P and compared with the reference kidney - anti kidney immune system two precipitates were formed with each of the Lancefield extracts and anti K/P. One of the two precipitates in each immune system coalesced with one immune precipitate of the kidney anti kidney system (aA). The other precipitate of the CM - P 13846 - anti K/P system coalesced with the dD precipitate while the second

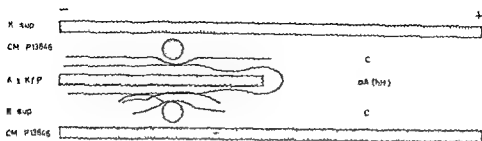


Fig 4

Schematic drawing of a combined comparative immuno electrophoretic analysis of the kidney antigens (K sup) and the Lancefield extract from the nephritogenic strain P 13846 using the anti kidney serum. The precipitate designations correlate with Fig 3. Serological relationship of the kidney *ad* and streptococcal *hl* precipitates is mentioned in the text.

CM - P 13846 - anti K | P system coalesced with the *ad* precipitate of the kidney - anti kidney system. No difference was noted in these precipitation patterns if an anti kidney serum was used which had previously been absorbed with human AB(+) erythrocytes.

Comparative immune electrophoretic analyses of the kidney - anti kidney immune system and intracellular preparations and Lancefield extracts of cell walls from the two aforementioned streptococcal strains were also performed. As may be seen in Fig 4 both the *ad* and the *hl* precipitates could be localized in the kidney - anti kidney immune spectrum. By means of a particular basin arrangement it could also be illustrated that the *ad* precipitate of the reference streptococcal spectrum coalesced with the *hl* precipitate of the reference streptococcal spectrum but no antibodies against the other kidney antigens were demonstrable in the anti IC+FC serum. Lancefield extracts of the other streptococcal strains containing the *h* factor developed a precipitate with the anti kidney serum which coalesced with the *ad* precipitate of the kidney - anti kidney system. When the lung antigen was electrophoretically separated and developed with anti K | P serum a precipitate was formed which coalesced with the *ad* precipitate of the kidney - anti kidney system.

DISCUSSION

The multitude of precipitinogens in intracellular materials from streptococcal strains belonging to Lancefield's groups A, C and G as illustrated by diffusion in gel methods is striking. Although no attempts were made to analyze the chemical structure of these antigens the present results give enough information to make a tentative characterization of some of the precipitinogens in the reference intracellular streptococcal antigen preparation. Thus it may be assumed that the *g* factor corresponded to the *F*₁ factor of Wilson & Wiley (1963) because of its wide distribution among different A, C and G streptococcal strains, its heat and trypsin stability and its electrophoretic position. The heat

TABLE 3

Summation of Results

Tentative Characterization of Streptococcal and Human Kidney Precipitinogens

Precipitinogenic factors	Properties
Reference streptococcal antigen	
a b g k	Related to human pathogenetic A C G strep strains
m	Specific to reference strep strain P 13846
f	Specific to strep Group A strains
h	Serologically related to human kidney antigen a and to a human lung antigen Common to strep Groups A C G
m	DNase inhibitor Common to strep Groups A C G
n	Mucopeptide Common to strep Groups A C G
q	E ₁ (Wilson & Wiley 1963) Common to strep Groups A C G
Human kidney antigen	
a	Serologically related to ref strep antigen h and a human lung antigen
c	Serologically related to a cell wall antigen from a nephritogenic strep strain (P 13846)
d	Serologically related to a cell wall antigen from a non nephritogenic strep strain (S 84)

and trypsin stable *n* factor might be correlated to the mucopeptide described by Krause & McCarty (1961) and which Rotta *et al* (1960) found could be released from streptococcal cell walls by ultrasonic treatment. Presence of streptococcal mucopeptide in IC materials may offer an explanation to the excellent antibody response obtained in the sheep if considered in relation to a recent report on the adjuvant property of mucopeptides (Holton & Schwab 1966). According to Table 2 factors *a b g* and *k* were only found in beta hemolytic streptococci which are considered to be primary human pathogenic. On the other hand the *a* and *b* factors were often difficult to demonstrate further more only three non human pathogenic strains were analyzed. The characterization of these factors as typical for human pathogenic streptococci is therefore highly tentative and additional streptococcal strains should be analyzed. The heat and trypsin labile factor *f* was only found in group A streptococci and might be regarded as a group A specific antigen not correlated to Lancefield's group specific carbohydrate as this latter substance resists trypsin treatment and 170 °C. The *c* and *d* factors were only found with certainty in the reference type 12 strain. Both factors were heat labile and trypsin stable like the T substance of Lancefield (1954). Although the immunoelectrophoretic position of the *h* also *c* responded to the electrophoretic position of

the T-substance from a type 17 streptococcus reported by Wilson & Wiley (1963) further analyses should be performed to establish the correlation between the T substance and the *c* or *f* factors. The *c*, *r*, *s* and *f* factors were found in all A, C and G streptococci tested. Whether these four factors are specific for the A, C and G streptococci or are also present in streptococci belonging to other groups cannot be interpreted from the present results. The trypsin stable *d* factor was found in most A, C and G streptococcal strains. Although the immuno electrophoretic position of the *dD* precipitation arc corresponded to the position of streptococcal polyglycerophosphate as described by Wilson & Wiley (1963) the *d* factor cannot be the polyglycerophosphate as antihodulus to this antigen were not detected in the anti IC+I C serum. The *h* factor could be demonstrated in all of the investigated A and C strains and also in the T 16 strain of group C but could not be demonstrated in the F 68 strain. The correlation between this factor and antigens derived from normal human kidney and lung will be discussed below. The *m* factor was found to be unevenly distributed among the A, C and G streptococcal strains. On the other hand IC materials from all strains which contained this factor also revealed DPNase inhibitory capacity. The immuno electrophoretic position of the *mV* precipitation arc corresponded to the *if* precipitate which was earlier shown by Holm & Kaijser (1966) to be correlated to the DPNase inhibitor and its antibody. Furthermore the results concerning heat and trypsin lability indicated that the *mV* precipitate might be attributed to the inhibitor anti inhibitor immune system. A summation of the characterization of these precipitinogens is presented in Table 3. It must be emphasized however that this is only a tentative characterization. Additional strains should be analyzed and purified antigens should be prepared in order to verify the trend of the present findings. In a forthcoming publication (Holm to be published) a further biochemical characterization of some of these factors will be presented together with results concerning the antigenic relationship between these factors and well known extracellular streptococcal factors such as streptolysin O, DPNase, B proteinase, streptokinase, DPNase etc.

The results concerning the antigenic relationship between human kidney antigen and streptococcal products showed that both intracellular material and I needfield extracts from cell walls from nephritogenic and non nephritogenic streptococcal strains belonging to the serologic groups A and C and the T 16 strain of group C all contained a heat and trypsin stable precipitinogen (the *h* factor) antigenically related to a human kidney precipitinogen. Furthermore this antigen was found to be related to a precipitinogen derived from normal human lung tissue. Immunologic cross reactions between lung and kidney preparations have been reported by several authors (e.g. Mellors et al 1955, Bjorklund 1956 and Kaplan 1964) and have been assigned to antigens present in the capillary vascular bed. In 1958 Kaplan reported that rabbits in

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STUDIES ON IMMUNOLOGICAL TOLERANCE TO LCM VIRUS

7 Adoptive Immunization of Virus Carrier Mice by Grafts of Normal Syngeneic Lymphoid Cells

By

JØRGEN HANNOFFER LARSEN and Mogens VOLKERT

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Billingham *et al* (2) demonstrated in 1956 that normal syngeneic lymphoid cells are capable of abolishing a state of immunological tolerance when transplanted into tolerant animals. This was later confirmed by the same group (3) and by Gowers *et al* (8). In the experiments described by these authors the state of tolerance was induced to allogeneic tissue and spleen cells, thoracic duct cells and peripheral blood leucocytes were also employed. Volkert (22) has shown that the same results could be obtained in another tolerance system: he found that normal spleen cells were able to abolish the tolerant state in LCM virus carrier mice.

As the role of the normal lymphoid cell is one of the fundamental problems in the question of tolerance, the effect of these cells in LCM virus carrier mice was reinvestigated using varying cell doses and long observation time. The effect of immune lymphoid cells has been described in our previous papers (21, 22, 23, 24, 25, 28). The results of the normal cell experiments will be recorded in the following.

MATERIAL AND METHODS

The tolerant LCM virus carrier mice were of the highly inbred C3H strain used in our previous experiments. The tolerance was induced by neonatal injection of LCM virus. Only female mice were employed.

The normal (unselected) donors used were of the same inbred strain and had never been in contact with LCM virus.

The preparation of cell suspensions, the transplantation, the serological and viral methods were the same described in our previous papers (2, 24, 25, 28).

The authors are indebted to Mrs B. Højer Simonsen for skilful technical assistance.

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EXPERIMENTAL

In the following experiments normal (unsensitized) lymphoid cells were taken from a pool of cells harvested from the spleens and lymph nodes of normal C3H female mice. The cells were transplanted into syngeneic virus carriers in doses of 10×10^6 , 100×10^6 , 200×10^6 and 1000×10^6 . After the transplantation blood samples were taken on days 10, 20, 30, 40-45, 60, 100, 140, 190 and 220. In addition for the 10×10^6 group blood samples were taken on day 420 after the transplantation for the 100 and 200×10^6 groups on days 370 and 420 after the transplantation and for the 1000×10^6 group on day 280 but not later than this. All mice survived in observation period of 190 days. After this time there were sporadic deaths of a few mice in each group. As is apparent from the figures these deaths after the 190th day did not however influence the experimental results.

The blood samples from each individual mouse were tested for virus and complement fixing antibodies. The blood was taken from the inner canthus of the eye. A small amount of heparin just enough to hinder coagulation was added to the blood for virus titration. Shortly after the blood was taken serial decimal dilutions were made and each dilution was injected intracerebrally into four white Swiss mice. The whole procedure was completed within an hour of the blood being taken. No heparin was added to the blood for the complement fixation test, these samples being allowed to coagulate. The serum was then separated after centrifugation at 3000 rpm for 20 minutes, inactivated on a water bath at 56°C for 30 minutes and stored at 4°C. The complement fixation tests were carried out once a week.

From the data obtained it was possible to distinguish five main types of experimental results (A, B, C, D and E) in the whole material. In Figs. 1 to 5 these five types are shown graphically. Each of the five pairs of curves indicates the course of the viremia and antibody titre in a single transplanted virus carrier mouse representative of the type. The A curves (Fig. 1) are from a mouse of the 10×10^6 group. The B, C- and D curves are shown in Figs. 2, 3 and 4 and are all from mice injected with 100×10^6 cells. The E-curves (Fig. 5) are from one in the 1000×10^6 group. In each group minor deviations from the curves shown were seen but these will be described in the text.

Control group. A fifth group of 10 C3H female virus carrier mice was used as control group. They were left untreated i.e. they were not transplanted. The virus titres of the blood and the antibody titres were determined as described above for the other groups. All the mice in this group had a course of the A type in Fig. 1. At no time did the virus titre fall below 10^{-2} and the complement fixing titres were <4 . These results are in accordance with previous findings in more than 100 untreated virus carrier C3H mice kept as controls in other kinds of experiments in this laboratory.

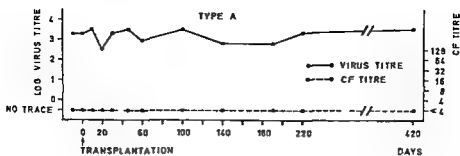


Fig 1

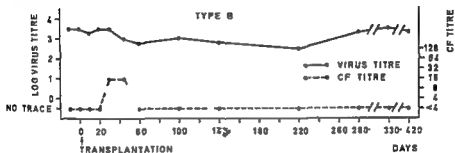


Fig 2

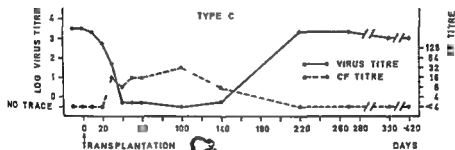


Fig 3

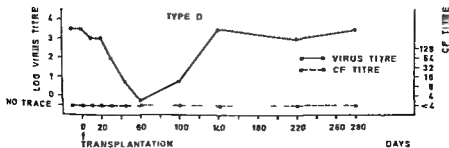


Fig 4

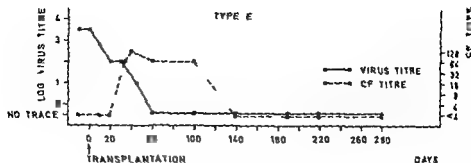


Fig. 1

Fig. 1—

Blood virus titres and complement fixing antibody titre of tolerant L3H virus carrier mice following transplantations of lymphoid cell from unsensitized genetic donors. Each figure indicates the data from one individual mouse representative of the group to which it belongs. The mouse in Fig. 1 has received 10×10^6 cells; those in Figs. 2 to 4 have received 100×10^6 cells and the mouse in Fig. 5 1000×10^6 cells.

The effect of 10×10^6 syngeneic lymphoid cells. In the following experiment a dose of 10×10^6 normal lymphoid cells isolated from the spleens and lymph nodes of syngeneic female donors was transplanted intraperitoneally to seven female L3H virus carriers. The blood virus titres and the complement fixing antibodies were followed at intervals for the next 420 days. The values for one of these mice are recorded graphically in Fig. 1. All the seven mice had a course of the A type. At no time did the virus titre of the blood fall below 10 and the antibody titres were < 4 . The course is identical with that of the untreated control group, i.e. no measurable adoptive immunization took place in these transplanted animals.

The effect of 100×10^6 syngeneic lymphoid cells. A group of 10 female L3H virus carriers was each transplanted intraperitoneally with a dose of 100×10^6 normal syngeneic lymphoid cells isolated from the spleens and lymph nodes of normal female donors. The blood virus titres and antibody titres were followed as described above.

From the data obtained in the individual mice three types of results could be seen in this group. In three mice the course was of the B type (Fig. 2). In these mice no virus titre below 10 was found but complement fixing antibodies were produced in all of them during the first two months after the transplantation. One month after the transplantation titres of about 16 were observed. However the antibodies soon disappeared and did not reappear. This means that a very short lived and weak adoptive immunization took place during the first two months after the transplantation, as indicated by the presence of complement fixing antibodies. It did not however influence the viraemia.

Eight further mice from this group had a course of the C type although the course in some mice differed slightly from the curves shown (Fig. 3). In all these mice the blood virus titres decreased and

the minimum titres were reached two to three months after the transplantation. In four mice the virus in the blood disappeared completely. In the other four mice the titres fell to between $10^{1.5}$ and 10^1 . The minimum titres were found in some mice for only a short time in a few at only one observation whilst in other mice the minimum titres were maintained at a constantly low level for several months. In all eight mice however the virus titres increased again and after one year all the titres were more than $10^{1.5}$ i.e. at the virus carrier level.

In these eight mice complement fixing antibodies were formed in small to moderate amounts (titres of 16-64 max.) during the first six months. Then they disappeared and did not reappear. In these mice a quite strong but temporary adoptive immunization took place as judged by both the temporary decrease of the viremia and by the presence of antibodies.

The remaining four mice of the 100×10 group had a course of the D type (Fig. 4). The minimum titres for these four mice were reached at 60 days after the transplantation and were of trace amounts in two mice and of titres of $10^{0.5}$ and 10^0 in the other two. In all four mice the blood virus titres hereafter returned to the virus carrier level. The antibody titres for these four mice were all negative (<4) during the whole observation period. This indicates that in these mice there was a temporary adoptive immunization as judged by the decrease in the blood virus titres but in spite of this no antibodies were formed.

The results for the whole 100×10 group show that a few mice will develop a short lived and weak adoptive immunization characterized by low titre antibodies but not influencing the viremia. In a few mice the induced immunization depresses the virus titres but does not cause antibody production. The main part of the mice will however develop a quite strong but temporary adoptive immunization which as long as it lasts influences the virus titres and leads to production of antibodies.

The effect of 200×10 syngeneic lymphoid cells. Nine female C3H virus carriers were transplanted intraperitoneally with 200×10 lymphoid cells isolated from the spleens and lymph nodes of normal syngeneic female donors. The viremia and the complement fixing antibodies were followed as described above.

Two types of results were seen in this group. Three mice showed a course of the C type (Fig. 3) not differing essentially from that described in the foregoing section on 100×10 cells. A temporary adoptive immunization took place. Six mice had a course of the F type (Fig. 5). The virus titres decreased to titres $\leq 10^{-1.0}$ to 60 days after the transplantation and stayed on this low level for the remainder of the 410 day long observation period.

Complement fixation and neutralization tests were in this group not carried out on individual samples but on a pool of serum from all members of the group. The complement fixation antibody titres were demonstrable from the third week and during the remaining observa-

tion period the titres varied from 64 to 128. The classification of this group is therefore made only according to the course of the viremia. The titres of the neutralizing antibodies in this group were followed for the first 70 days after the transplantation. These antibodies appeared in the second week after the transplantation, reached maximum values six weeks later with a log neutralization index of 3.0 and then decreased again. Seventy days after the transplantation the log index was 1.9. Further tests were not made.

The results from these six mice revealed a complete and long lasting abolition of the tolerant state, as is seen after transplantation of a sufficient number of immune cells.

The effect of 1000×10^6 lymphoid cells. A group of seven female C3H virus carriers was transplanted intraperitoneally with 1000×10^6 lymphoid cells isolated from the spleens and lymph nodes of normal syngeneic female donors. The viremia and complement fixing antibodies were followed for the next 270 days.

From the course of the viremia and antibody titres in the individual mice it was possible to distinguish two types of response in this group. Four mice had a temporary adoptive immunization (type C, Fig. 3). The minimum titres of all four mice were below 10. In all these mice the virus titres again increased to virus carrier values ($>10^5$). Three mice showed however a permanent adoptive immunization (type F, Fig. 5). Minimum virus titres of no trace were reached two months after the transplantation and stayed at this low level for the remainder of the observation period (280 days).

Complement fixing antibodies appeared four weeks after the transplantation. In all mice of this group antibodies were present. They reached maximum titres (32–128) 40 days after the transplantation. During the following period they dropped slowly to low levels or disappeared.

Table 1 contains an outline of the four groups described here and also of the control group of non transplanted virus carriers.

TABLE 1

Survey of the Course of the Viremia in Female C3H Virus Carrier Mice Transplanted with Varying Numbers of Lymphoid Cells Isolated from the Spleens and Lymph Nodes of Normal Syngeneic Female Donors together with a Control Group of Non Transplanted Virus Carriers

Cell number transplanted	Number of mice in the group	Type A	Type B	Type C	Type D	Type F
10×10^6	7	7	0	0	0	0
100×10^6	15	0	3	8	4	0
500×10^6	9	0	0	3	0	6
1000×10^6	7	0	0	4	0	3
Control	15	15	0	0	0	0

DISCUSSION

It is generally believed that adoptive immunization of tolerant animals is brought about by a repopulation of the recipients with the living immunologically competent cells from histocompatible (= syngeneic) donors and by the immunity conferred by these cells. However in accordance with the experimental evidence presented by *Väkela & Mitchison* (14) and by *Celada* (a) the recipients seem to possess a barrier to the repopulation with transplanted syngeneic cells or/and to the function of these cells. As the injected cells are compatible to their new host they should share the fate of the host's own cells. It therefore seems logical to assume that this barrier is caused by the same mechanism which must be operative in checking the number and the function of the lymphoid cells in a normal mouse. Unfortunately this mechanism is completely unknown but it has been suggested that there might be a sort of competition between the lymphoid cells. This could be merely a question of *Lebensraum* but such phenomena as contact inhibition, gamma globulin feedback, humoral depression and other factors might also play a role.

Where mice tolerant to the LCM virus are concerned previous experiments in this laboratory (23-24) have shown that to obtain a detectable adoptive immunization a minimum of $5-10 \times 10^5$ lymphoid cells from immunized donors has to be injected into the virus carrier mice. The $5-10 \times 10^5$ cell doses are about 1/40 of all the cells contained in the spleen of an immunized donor. If one can judge from results of experiments in which other antigens than the LCM have been used as immunizing antigens in mice (9-11) the $5-10 \times 10^5$ cell dose mentioned above should contain about 10^3 to 10^4 sensitized cells. The mean generation time for immunologically competent cells according to *Vossel & Väkela* (15) and to other investigators (4-10-13) is about eight to twelve hours. An uncontrolled growth of much smaller amounts of sensitized cells than those contained in the minimum active cell dose should therefore multiply in the course of a few days to give a huge amount of immunologically active cells and cause a clear-cut immune response. The fact that such a reaction is not detectable when cell doses of less than 5×10^5 cells are injected strongly supports the hypothesis that a control mechanism in the host restricts the growth or the function of the injected cells.

The effect of the control mechanism on sensitized cells seems however to depend on the number of cells injected and on the antigenic stimulation the cells receive. When the cell dose is high enough and the antigenic stimulus is very strong the cells will not only survive and function but seem to be able to overcome the control mechanism completely.

This phenomenon is apparent from the fact that a dose of about 100×10^5 lymphoid cells from immune donors is about only one fifth

from our experiments that a kind of balance exists between cells and control mechanism. When small cell numbers are injected the control mechanism completely suppresses the cells. On the other hand in cases where large doses of sensitized cells are employed the cells might outbalance the control mechanism. The influence of cell number on the outcome of the balance might perhaps also play a role a long time after the cells have been transferred. Perhaps in cases in which the population of cells never reaches a high level the balance might tip to the advantage of the control mechanism in the course of time and then the immunological activity and perhaps the proliferation and life of the cells will be brought to an end. In cases where sensitized cells are employed this might never happen simply because these cells are highly susceptible to the antigenic stimulus they receive when injected into the virus carrier recipients. As pointed out above this stimulus probably supports the colonization. As a result a large cell population is likely to be produced in all cases in which the initial cell dose has been high enough to overcome the immediate effect of the control mechanism. However normal cells are probably not stimulated to the same degree and judging from the antibody titres obtained and from the influence on the virus in the recipients the number of immunologically active cells derived from the injected normal cells will never reach a high level. In such cases the control mechanism might have a chance to suppress the cells even after they have settled and the return of tolerance might be the result.

The phenomenon of a change from tolerance to immunity and back to tolerance has also been observed under other experimental conditions with the LCM virus. *Hannover Larsen* (12) has shown that this happens in infected infant mice if the infection is postponed until the end of the period in which mice are fully susceptible to the induction of tolerance. *Hannover Larsen* found that babies infected at the age of four to nine days can develop tolerance to the virus but this tolerance is only temporary. In the course of time antibodies will appear and this is associated with a drop in the blood virus titres. However later a full degree of tolerance often recurs spontaneously. These findings also point to the existence of a balance between immunologically reactive cells and control mechanism. These results will be presented and discussed in a later paper.

Chase (6) has reported that in adoptive immunization of guinea pigs with sensitized cells the ability to produce antibodies and the state of hypersensitivity conferred by the same batch of cells did not run parallel in different animals. In some of the recipients the ability to produce antibodies largely superseded the hypersensitivity in others the reverse was the case. *Chase* points out that these results indicate that the antibody production and the hypersensitivity are caused by different cells. Other investigators (1, 7, 16, 17, 19) have also obtained experimental evidence pointing in the same direction. From our experi-

ments it is apparent that the production of complement fixing antibodies and the decline of the blood virus titres similarly do not run parallel. We have mice in which there is a demonstrable antibody production simultaneous with constant high blood virus titres and on the other hand we have mice in which the virus disappears in the blood in spite of the fact that no complement fixing antibodies can be detected. In a previous paper (27) we have pointed out that we have many other data which indicate that the elimination of the virus in adoptively immunized virus carriers is most probably caused by a direct cellular function and not by the antibodies. The results presented in this paper support this view but also seem to support the idea that different cells are responsible for the different parts of the very complex immune mechanism. Moreover the effect of the control mechanism on these different kinds of cells seems to vary and to differ making an independent action of one or other group possible.

SUMMARY

The effect of adoptive immunization with normal (unsensitized) syngeneic lymphoid cells on tolerant LCM virus carrier mice using varying cell doses has been investigated.

10×10^6 cells gave no measurable adoptive immunization. 100×10^6 cells gave a weak and short lived adoptive immunization in some mice in others a stronger but still temporary adoptive immunization. 200×10^6 and 1000×10^6 cells gave a quite strong adoptive immunization in all mice but led to a long lasting abolition of the tolerant state in only some of the mice.

In many mice there is no parallelism between the two measured functions of the adoptive immunization the antibody formation and the virus elimination. This indicates that two functionally different systems of immunologically reactive cells are involved.

The problem of cell dose with particular regard to a homeostatic cell control mechanism of the animal is discussed.

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STUDIES ON THE CELL TOXICITY AND SPECIES SPECIFICITY OF PURIFIED STAPHYLOCOCCAL TOXINS

By

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In the efforts to understand the pathogenesis of staphylococcal infections many approaches have been tried. Several papers offer evidence which strongly suggests that the staphylococci owe at least part of their pathogenic capacity to extracellular toxins and enzymes (6-24). Factors which exert cytotoxic action on human cells are of course of special interest.

To study this problem many authors have used cell cultures as experimental models. Most works concern alpha hemolysin which has been reported to have a cytotoxic effect on various cell species such as rabbit smooth muscle and kidney cells (1-7, 23), mouse kidney cells (7), chick embryo cells (21), guinea pig skin (17), human amnion kidney and skin cells (1-7, 17) as well as Ehrlich ascites carcinoma cells (18).

In some reports the cytotoxic effect of other toxins has also been investigated. *Korbecki et al.* (16) found strong cytotoxic activity by both alpha and beta hemolysins on KB and monkey kidney cells. Deoxyribonuclease had feeble activity on KB cells and stronger on HEP-2 cells. Neither leucocidin nor staphylokinase were cytotoxic for the cell cultures studied.

According to some authors no cytotoxic effect was observed when purified enterotoxin B preparations were tested on HEP-2, HeLa and human heart cells (20) or monkey kidney cells (14). This disagreed with the findings of other authors. *Guerin et al.* (9) found a cytopathogenic effect on chick embryonic heart fibroblast and *Schaeffer et al.* (22) a cytotoxic effect on cell cultures of human embryonic intestine.

The toxin preparations used are however often crude or unsatisfactorily characterized. Furthermore earlier reports have not systematically dealt with the effect of various toxins on human cells.

In this report the effect with regard to cell toxicity and species specificity was followed in fractionation experiments whose primary aim was the study of factors toxic to human cells. The results were

also considered in relation to the effect on other cells. The purity of all preparations was immunologically characterized. As alpha beta and delta hemolysin in this work were tested on cells other than erythrocytes they were named alpha beta and delta lysis resp.

MATERIALS AND METHODS

Strains *Staphylococcus aureus* strains 5C and 196F (3) were used for toxin production.

Preparation of crude toxin for fractionation Crude toxin was produced on solid medium covered with cellophane as described earlier (11). The pooled extracts were concentrated according to Kohn (15).

Toxin assay Coagulase, enterotoxin B, leucocidin, hyaluronidase, lipase, alkaline phosphatase, staphylokinase and DNase were determined as described in previous papers (10, 11).

Enterotoxin A assay 1) *In vitro* The preparations were tested for enterotoxin A in gel diffusion (25) with a purified anti-enterotoxin A serum kindly supplied by Dr. F. P. Casman. 2) *in vivo* The cat test was used as described in a previous paper (12) except that the preparations were not boiled before the injection but neutralized with a serum containing high anti-alpha and anti-beta lysis titres (Burroughs Wellcome Ltd. no. RA 358).

Proteolytic enzyme Plates for testing proteolytic enzyme activity were prepared using matrices for basin plate technique according to Ouchterlony. The agar (1 per cent) contained 1 per cent gelatine. The basins were filled with the samples and the plates were incubated at 37°. After 12-18 hours the plates were flooded with saturated ammonium sulphate.

In the presence of proteolytic enzyme activity a clear zone appeared around the basin.

Alpha beta and delta lyses were determined as described earlier (10) except that all dilutions for beta lysis were performed in phosphate buffered saline (pH 7.0) containing 10^{-3} M MgSO₄ according to Haque et al. (13). So rabbit sheep and human erythrocytes were used for the titration of alpha beta and delta lyses resp.

All titrations were read both after 1 hour at 37° and after 1 hour at 4°.

Cell cultures A calf serum adapted strain of HeLa cells originally adapted to horse serum according to Mandel (19) was grown in Hank's salt solution enforced with 0.1 per cent yeast extract and 0.1 per cent lactalbumin hydrolysate and containing 20 per cent calf serum. KB cells were grown in the same medium.

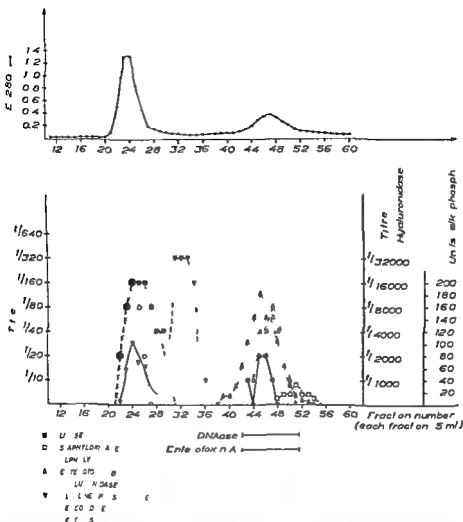
Primary human kidney cells and foetal colon cells were grown in Eagle's medium (2) with 10 per cent calf serum. Primary cultures of calf cells were prepared from trypsinized kidneys of newborn calves and grown in Hank's solution with 0.5 per cent lactalbumin hydrolysate and 10 per cent calf serum.

Primary cultures of cynomolgus monkey kidney cells were grown in Eagle's medium with 10 per cent calf serum. After outgrowth the cultures were changed to Eagle's medium without serum. The cells were grown in plastic petri dishes at 37° in a humidified atmosphere of 5 per cent CO₂ in air. All media contained penicillin (100 U/ml) and streptomycin (100 µg/ml).

Granulocytes adherent to cover glasses were prepared according to Cliftone (8).

Cell toxic studies Before use all toxins were calibrated to 0.02% sodium phosphate buffer containing 0.1 M NaCl, pH 7.2-7.4.

Primary assay of cell toxicity Plate cultures with the cells to be tested were overlaid with five ml agar containing 0.17 per cent NaHCO₃, 4 per cent calf serum and 0.95 per cent special agar Noble (Disc 1) in Eagle's medium. When the agar had solidified it was cut with an agar gel cutter and the agar core removed by gentle suction. The resulting wells were then filled with the toxin material which was serially diluted in Eagle's medium. After incubation at 37° in 5 per cent CO₂ in air for the intended time usually 2 hours the toxin was removed by suction and the plates covered with 5 ml of the same agar overlay containing neutral red at a concentration of 1/20000. The plates were usually read after 6 and 10 hours. Cell death was roughly indicated by loss of ability to stain with neutral red.



F 1

Gel filtration of 10 ml crude toxin from strain S6 through Sephadex G-100. The extinction at 280 mμ as well as the localization and titres of different activities are indicated. DNase and enterotoxin A present in the low titers are indicated below.

Radioisotope studies. The toxin effect was also studied following the release of P₃ previously incorporated into plate cell cultures. The plate was washed once with phosphate buffered saline (PBS) and 1 μCi of carrier free sodium orthophosphate (Radiochemical Centre, Amersham, England) in 5 ml phosphate free Eagle medium added. After 4 hours the medium was sucked off the plate washed once with PBS and 1 μCi Eagle medium added. After a further 24 hours the medium was removed the cell washed 4 times with PBS and the toxin to be tested appropriately diluted in Eagle medium added to the 11 in 5 ml portions. Samples of 0.1 ml were withdrawn at 10 min intervals and the radioactivity counted in a thin end window Geiger tube.

Immunization. Gel and thin layer electrophoresis was performed as described earlier (12).

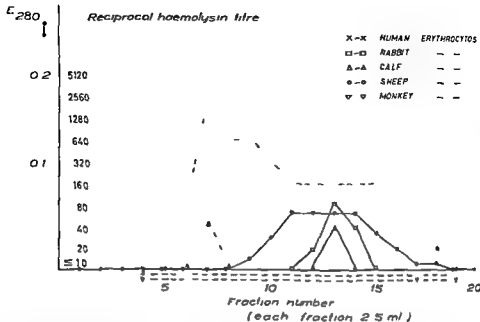


Fig 9

Preparative electrophoresis of a 196F alpha lysin peak from gel filtration on a Sephadex C-5 column (void volume 60 ml). Two ml of the material was run towards the cathode at 500 V for 24 hours in 0.05 M barbital buffer pH 8.2. The hemolytic effect on human rabbit calf sheep and monkey erythrocytes is indicated. Broken lines indicate hot cold phenomenon.

Gel filtration was performed on Sephadex G-100 (Pharmacia Fine Chemicals, Lppsala, Sweden) in a glass column 45 x 35 cm. The elution buffer was 0.05 M NaCl containing 0.1 M NaCl pH 7.2. Elution was carried out at 4 ml/cm/hour and the effluent was collected in fractions of 5 ml.

As shown earlier (10) Sephadex G-100 separates crude S6 toxin into three main peaks as is illustrated in more detail in Fig 1. Crude toxin from strain 196F was fractionated in a similar way. Beta lysin and proteolytic enzyme which were produced by strain 196F but not by strain S6 appeared in the third peak. Enterotoxin A which was produced only in small concentrations by strain S6.

Preparative electrophoresis was performed on a vertical column (20 x 20 cm) packed with Sephadex C-50 (Pharmacia Fine Chemicals) and void volume 1 ml. The column was cooled to 4°C with a cooling jacket. The fraction volume was 5 ml. Barbital buffer 0.05 M pH 8.2 was used.

Preparative electrophoresis was used for further purification of the alpha lysin materials. Active material was always pooled concentrated (15) and dialyzed before the electrophoresis.

Protein concentration was estimated routinely by measuring the optical density at 280 mμ in a Beckman DU spectrophotometer.

Toxin and Enzyme Preparations

alpha lysin. Two preparations of alpha lysin were tested. 1) The first was the fraction from the Sephadex C-100 eluate of the strain S6 showing the highest titre. As is evident from Fig 1 this material was contaminated with leucocidin, enterotoxin B, DNAase and typhoid kinase. 2) The second was a 196F alpha lysin peak from gel filtration further purified by preparative electrophoresis as shown in Fig 9. By this procedure the alpha lysin was partially separated from beta lysin. Besides beta lysin small amounts of enterotoxin A could be shown by the cat test. No proteolytic enzyme or leucocidin activity was found.

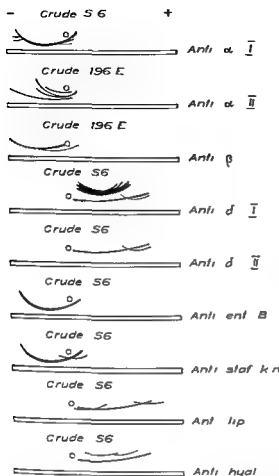


Fig 1

Schematic representation of immunoelectrophoretic analysis of immunoprecipitates prepared with purified toxin preparations. Anti α I represents S6 material from second peak of Sephadex C-100 anti α II 196E material after purification by Sephadex G-100 and preparative electrophoresis. Anti δ I represent S6 material from first peak of Sephadex C-100 and δ II S6 material after purification by Sephadex G-100 and preparative electrophoresis. Precipitation arcs present in pre-immunesera are not illustrated in this fig.

An immunoelectrophoretic analysis of the two preparations is given in Fig 3. *Beta lysin*. This toxin tested a material from the second peak of gel filtration of the strain 196E purified by electrophoresis. No other activity was found in the preparation though two precipitation arcs developed when crude 196F toxin was developed by anti beta lysin as shown in Fig 3.

Delta lysin was tested against the top fraction from the first peak after gel filtration on Sephadex C-100 of toxin from the strain S6 or 196E, delta I in fraction S6 purified from lipase and alkaline phosphatase in preparation of electrophoresis. The material was run toward the anode at 500 V for 4 hours in 0.05 M barbital buffer pH 8.7. A detailed study on the purification of delta I as well as on the antigenic properties will be published separately. An immunoelectrophoretic analysis of the two preparations is given in Fig 3.

Lipase. The fraction designated as the first of the anti α peak of the strain S6 further purified by preparative electrophoresis as described under delta I. The purified product was not perfectly separated from antigen present in the delta lysin fractions as is shown in Fig 3.

Hyaluronidase was taken from the electrophoretic experiments of strain S6. The material contained antigen present in both lipase and delta I preparation (Fig 3).

Enterotoxin B. Immunologically pure enterotoxin B was prepared by means of gel filtration and preparative electrophoresis as has been published separately (12).

Enterotoxin A has as yet not been adequately purified

Staphylokinase was further purified from gel filtration (Fig. 1) by preparative electrophoresis towards the cathode at 500 V for 24 hours in 0.05 M barbital buffer pH 8.2. After this procedure the enzyme was present only in titres of 1/4. The preparations did not contain any alpha lysin or leucocidin. A small amount of enterotoxin B was however present (Fig. 3).

Alkaline phosphatase, *DNAase*, *proteolytic enzyme* and *leucocidin* electrophore is destroyed the activity of these enzymes preventing further purification.

EXPERIMENTS AND RESULTS

Effect of Crude Toxin

The toxin enzyme spectra of the free supernatants of the two strains S6 and 196E are shown in Table 1. Although these strains are both coagulase positive in slide and tube tests according to Duffie (4) no coagulase activity could be found.

TABLE 1
Toxin Enzyme Spectra of Staph. aureus Strains S6 and 196E

Toxin tested	S6	196E
Coagulase	—	—
enterotoxin A	+	+
enterotoxin B	+	—
alpha lysin	+	+
beta lysin	—	+
delta lysin	+	+
leucocidin	+	—
hyaluronidase	+	+
lipase	+	—
alkaline phosphatase	+	+
proteolytic enzyme	—	+
DNAase	+	—
staphylokinase	+	—

TABLE 2
Effect of Crude Toxin from Strains S6 and 196E on Different Cell Types

Cell type	S6	196E
HeLa cells	1/20	1/40
Monkey kidney cells	1/5	1/20
Calf kidney cells	1/80	1/160
IB cells	1/5	1/10
Human erythrocytes	1/0	1/100
Monkey erythrocytes	1/40	1/40
Calf erythrocytes	1/100	1/1280
Guinea pig erythrocytes	1/1000	1/2060
Sheep erythrocytes	1/80	1/5120 (1/40000)

Where the titres rose more than 2 steps after 1 hour at +4 °C the values are given in brackets in addition to titres after 1 hour at 37 °C.

The cytotoxic effects of the crude preparations were tested on erythrocytes and cells from various mammalian species as shown in Table 1. As this table shows both strains exerted a toxic effect on the cells tested. Higher titres were obtained with blood cells than with cell cultures from corresponding species. Further the effect on blood cells from various animals varied considerably.

Effects of purified toxins Parallel to the purification procedure all fractions were tested on erythrocytes from man, monkey, calf, sheep and rabbit, on granulocytes from man as well as on human, monkey and calf kidney cells. Fractions with hemolytic or cytotoxic effect in the primary assay were further investigated in radioisotope studies on human, monkey and calf kidney and sometimes other cells.

Alpha lysin As shown in Table 3 none of the preparations had any effect on red blood cells from man and monkey but elicited a strong action on calf and rabbit erythrocytes. The effect of alpha lysin of the strain 196E on sheep erythrocytes had hot cold character and was probably due to beta lysin present. No effect on human granulocytes could be demonstrated with this preparation. Alpha lysin from the strain S6 which lacked beta lysin had no or a very slight effect on sheep erythrocytes but attacked human granulocytes in a special way without lysis of the cell but with nuclear swelling and nuclear lysis. The table also shows that alpha lysin had a toxic effect on calf kidney cells but did not affect human and monkey kidney cells under the conditions studied. The effect of alpha lysin on tissue culture cells was also studied in repeated experiments by following the release of P^{32} from cells. The results of one such experiment are given in Fig. 4 which shows that alpha lysin had a greater effect on calf kidney cells than on human kidney cells. When cruder alpha lysin preparations were used human kidney cells were also attacked. The effects of alpha lysin on KB and HeLa cells and monkey kidney cells were usually less marked than on calf cells.

TABLE 3

Effect of alpha Lysin I preparations on Erythrocytes and Tissue Culture Cells from Various Species

Cell type	as shown from	
	S6	196E
Human erythrocytes	<1/10	<1/10
Monkey erythrocyte	<1/10	<1/10
Calf erythrocyte	1/40	1/40
Rabbit erythrocyte	1/30	1/80
Sheep erythrocyte	1/20	<1/10 - 1/160
Human kidney cell	<1/3	<1/2
Monkey kidney cells	<1/3	<1/2
Calf kidney cell	1/3	1/4

When two titre values are indicated cold phenomenon is indicated

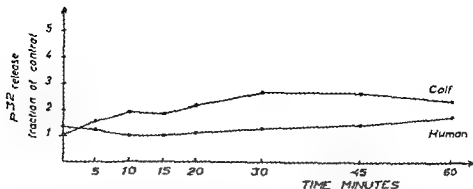


Fig. 4

Effect of alpha lysin on human and calf cells. Cells were labelled with P^{32} as described in Material and Methods. The hemolytic titre of the incubated alpha lysin preparation was 1/64. Results are given as fraction of the control culture from the same time.

Beta lysin. Beta lysin is characterized by so called hot-cold hemolysis (6). As shown in Table 4 this toxin acted mainly on sheep erythrocytes and had no effect on red blood cells from man, monkey or rabbit or on human granulocytes. A weak effect was noted on calf blood cells. The preliminary tissue culture studies showed that beta lysin with a hemolytic titre of 1/2560 had no visible cytotoxic effect on human, monkey or calf kidney cells. In the radioisotope studies beta lysin with a hemolytic titre of 1/25 had no effect on human and monkey kidney or all cells and only a very slight effect on calf kidney and Mandel cells.

TABLE 4
Effects of β Lysin on Erythrocytes and Tissue Culture Cells

Cell type	37	+37 and +4°C
Human erythrocyte	<1/4	<1/4
Monkey erythrocyte	<1/4	<1/4
Calf erythrocyte	<1/4	1/1
Rabbit erythrocyte	<1/4	<1/4
Sheep erythrocytes	<1/2560	1/2560
Human kidney cells	<1/2	nt
Monkey kidney cell	<1/2	nt
Calf kidney cells	<1/2	nt

nt = not tested

Delta lysin (= hemolysin). As Table 5 shows delta lysin had a strong effect on human, monkey and rabbit erythrocytes and a weak one on calf and sheep erythrocytes. Corresponding hemolytic effect on erythrocytes from the various species could also be demonstrated with delta lysin after additional purification in preparative electrophoresis. Human granulocytes were quickly lysed after exposure to delta lysin. Of the tissue culture cells only human and monkey kidney cells were affected.

TABLE 3

Effect of δ Lysin on Erythrocytes and Tissue Culture Cells

Cell type	δ Lysin from Sb	1961
Human erythrocytes	1/80	1/40
Monkey erythrocytes	1/160	1/20
Calf erythrocytes	1/90	<1/90
Rabbit erythrocytes	1/80	1/20
Sheep erythrocytes	1/10	<1/20
Human kidney cells	1/4	1/4
Monkey kidney cells	1/2	<1/9
Calf kidney cells	<1/2	<1/2

as demonstrated by vital staining. Delta lysin was also studied with the isotope method and one such experiment is shown in Fig. 5. The results clearly show that delta lysin had a pronounced effect on the release of P^{32} from human kidney cells whereas the effect on calf kidney cells was weak. The release of P^{32} from monkey kidney cells lay between that of human and calf kidney cells and was weaker than that with comparable titres of alpha lysin.

Lipase The tested material which had a lipase titre of 1/10 had no

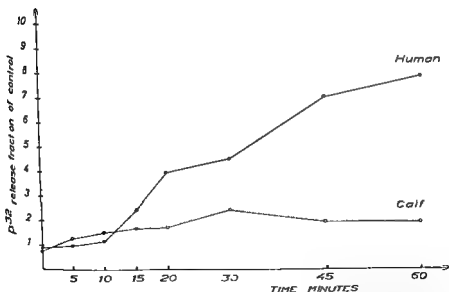


Fig. 5

Effect of delta lysin on human and calf cells. Cells were labelled with P^{32} as described in Materials and Methods. The hemolytic titre of the inoculated delta lysin preparation was 1/16. Results are given as fraction of the control culture from the same film.

visible effect on erythrocytes or tissue culture cells from the species used in this study

Hyaluronidase No visible effect was found on erythrocytes or tissue culture cells when hyaluronidase was used in a titre of about 1/20000. A slight effect was found by isotope studies with titres of 1/1280.

Enterotoxin B Enterotoxin B had no visible effect on erythrocyte and tissue culture cells. Tissue culture cells were also tested by the isotope method with enterotoxin B preparation in a titre of 1/32 but no increase in the release of P^{32} was observed. An attempt to infect tissue cultures from human embryonic colon resulted in growth of fibroblasts which were not injured by enterotoxin B.

Staphylokinase present in titres of 1/4 after gel filtration and preparative electrophoresis gave no effect on the cells tested. This was true also for the isotope studies where the material however was diluted further four times.

DISCUSSION

This report describes the effect of alpha, beta and delta lysin lipase, hyaluronidase, enterotoxin B and staphylokinase prepared to different degrees of purity on cells from various species. One of the strains S6 and 1961 depending on capacity was used for toxin production. Alkaline phosphatase, DNA ase, proteolytic enzyme and leucocidin which were produced by both strains were not studied in detail because their activity was lost during the purification procedure. Enterotoxin A has so far not been prepared free from alpha and beta lysin. Though both strains were coagulase positive no free coagulase could be found in the supernatants. The reason for this is unclear.

In order to remove impurities all purified preparations were used for immunization and the sera were analyzed against crude toxin in immunoelectrophoresis.

Only enterotoxin B was purified to a state of immunological purity. Of the lysins beta and delta lysin were highly purified whereas alpha lysin was purified to a lesser degree. This is further substantiated by the presence of other active enzymes in these preparations (Figs 1 and 2). Staphylokinase was contaminated with enterotoxin B lipase with antigens present in the delta lysin preparations and hyaluronidase with antigens present in both lipase and delta lysin preparations.

All fractions were tested on red blood cells from five species and tissue culture cells from at least three species. The effect on human granulocytes was also tested. The species specificity of the different fractions is partly defined since human erythrocytes are used for the titration of delta lysin, rabbit erythrocytes for alpha lysin and sheep erythrocytes for beta lysin. The cytotoxic effect is indicated by lack of vital staining after a two hour exposure of tissue cultures to toxin. It turned out to be rather insensitive with titres mostly 20-30 times

lower than those obtained in the corresponding erythrocyte tests. The changes were also difficult to interpret in some cases. The isotope method was found to be far more sensitive. Human erythrocytes were attacked only by delta lysin and it was found that lipase and alkaline phosphatase had no effect.

Human granulocytes were injured by delta lysin and also by a fraction from strain S6 containing alpha lysin, leucocidin, enterotoxin B, DNAase and staphylokinase. However, the same cytopathogenic difference was noticed between these two preparations as the one observed by Gladstone between delta lysin and leucocidin (8). As enterotoxin B, DNAase and staphylokinase were found to be noncytotoxic and human leucocytes are known to be very resistant to alpha lysin (2), the effect of the latter preparation was possibly due to leucocidin. Because of the difference in cytotoxic picture, no titres were given for human granulocytes.

Human kidney cells were attacked by the delta lysin fractions and to a less degree by preparations from the second gel filtration peak of strain S6 containing chiefly alpha lysin. They were very slightly influenced by purified alpha or beta lysins from 196F in comparable titres. This difference between the two alpha lysin preparations is unclear. As leucocidin as well as alpha lysin of strain S6 were lost when purified by preparative electrophoresis, the effect could not be studied further, but leucocidin was shown by Korbecki *et al* (16) to have no effect on KB, H Ep 2 or L cells. The feeble effect of hyaluronidase was probably due to contamination with delta lysin.

In other reports, alpha lysin has been shown to be cytotoxic for human skin explants (17), human amnion cell culture (18), KB cells (16). The experiments, however, vary considerably with regard to time of exposure and purity of the toxins and a comparison is therefore difficult. The results in this paper support the opinion that enterotoxin B is noncytotoxic to human cells (20) although Schaeffer *et al* (22) describes a cytotoxic effect after two days' exposure of human embryonic intestine in tissue culture.

Our findings with staphylokinase agree with those of other workers (16).

HeLa cells seemed to be more sensitive than human kidney cells in our experiments as it was strongly affected not only by delta lysin but also by alpha lysin and although slightly by beta lysin. This shows the importance of the choice of cell line.

KB cells were not injured by beta lysin in these experiments which disagrees with the findings of Korbecki *et al* (16) who however exposed their cells for 48 hours.

Of cells from other species, monkey and calf kidney cells were attacked both by delta and alpha lysin. This was however not in accordance with the results on blood cells since monkey erythrocytes were not hemolyzed by alpha lysin. Calf kidney cells were also damaged

by beta lysin. The same was true for calf erythrocytes which were lysed in the typical hot cold manner though to a much less degree than sheep erythrocytes.

Of all toxins tested the most interesting results concern delta lysin. It is obvious that this toxin is active against cells from a wide range of species. With respect to human cells this effect was however more pronounced than the effect of other toxins tested. It might be that delta lysin has been neglected as a potential virulence factor of staph aureus in human disease.

SUMMARY

The celltoxic effect of partly purified staphylococcal alpha lysin beta lysin delta lysin hyalase hyaluronidase enterotoxin II and staphylokinase was studied in short term experiments on erythrocytes and cell lines from different species as well as on human granulocytes. Delta lysin was shown to exert a strong toxic action on human erythrocytes human granulocytes and human kidney cells. The effect of alpha lysin on the same cells was much less pronounced. On the other hand alpha lysin exerted a stronger effect on calf cells than delta lysin. The other toxins and enzymes studied could not with any certainty be correlated to any cytopathogenic effect at 37°.

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ANTIGENIC STUDIES ON MICROCOCCUS STRAINS

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The taxonomic position of the micrococci is still very confusing. Two main features seem, however, to have been accepted by recent authors. First, micrococci can most reliably be distinguished from staphylococci by their inability to attack glucose anaerobically (2, 5, 11, 19). The validity of this distinction has lately been supported by investigations on the DNA base composition of members of the two genera (4, 1). Second, the genus *Micrococcus* can be subdivided into two groups based upon the ability or inability to attack glucose aerobically (1, 5).

Apart from this subdivision, there is little agreement on the classification of micrococci into species or groups, of which a large number have been described. In recent years Kocur & Martinec (15) have recommended that the genus *Micrococcus* should have only 6 species and 2 varieties. Baird Parker first recognized 7 subgroups (2), later 8 (3). Evans (6), like Bohacek *et al.* (4), is of the opinion that at present only two species, *i.e.* *M. luteus* and *M. roseus*, should be recognized, as others lack the homogeneity that should characterize valid species.

The antigens of the members of the genus *Micrococcus* are unknown. A study of micrococcal antigens, including their comparison with staphylococcal antigens, which are relatively well known, might contribute to the understanding of the micrococci. The present investigation has been carried out on a selected but limited material of culture collection strains classified as micrococci.

MATERIAL AND METHODS

Strains

Twenty *Micrococcus* strains were obtained from the Czechoslovak Collection of Microorganisms through the courtesy of Dr. M. Kocur. The strains had been selected so as to represent differences in biochemical activities and at the same time to represent some of the formerly recognized species. They had been classified according to the scheme of Kocur & Martinec (15). The material is listed as follows:

- 7 strains of *M. luteus* (CCM 810, 144, 1335, 210, 657, 696, 310)
- 5 strains of *M. roseus* (CCM 146, 491, 106, 861, 147)
- 4 strains of *M. conglomeratus* (CCM 740, 547, 84, 757)
- 4 strains of *M. varians* (CCM 579, 894, 719, 1176)

The strains were examined for colony and pigment formation on blood agar plates, cell morphology in Gram preparations from blood agar cultures, production

of catalase and coagulase acid production from glucose aerobically and anaerobically and typability with *Staph aureus* phages. None of the strains produced coagulase or was typable by phage. All produced catalase. Two strains (*M conglomeratus* 757 *M varians* 529) undoubtedly produced acid from glucose anaerobically (19). None of the *M luteus* or *M roseus* strains attacked glucose aerobically whereas all the *M conglomeratus* and all the *M varians* strains except *M varians* 719 did so.

The two strains which attacked glucose anaerobically could not therefore be accepted as micrococci but were probably staphylococci. The third irregular strain appeared to belong to another *Micrococcus* species than that in which it had been placed. These three strains are included in the antigenic study for comparison. A number of control strains of *Staph aureus* and *Staph epidermidis* have also been examined.

Methods

All the *Micrococcus* strains as well as the control strains were examined in *Micrococcus Staph aureus* and *Staph epidermidis* immune sera by slide agglutination, ring test and agar gel precipitation and haemagglutination of sensitized normal and tanned sheep erythrocytes.

Immune sera were obtained by intravenous injections of formalin killed bacteria in rabbits (16). Factor sera were produced according to the method of Oeding. Agglutination was performed on slides using live bacteria. For technical details see Naukenes (10) and Hofstad (12).

Ring test precipitation was carried out with the supernates of saline extracts of the bacteria against undiluted immune sera. The agar gel diffusion technique as described in (11). Eighteen hr live nutrient agar cultures of the strains were tested against undiluted whole sera. Polysaccharide A (9), polysaccharide 263 (13), protein A (7) and antigen D (14) from *Staph aureus* and the wall teichoic acid from *Staph epidermidis* strain T 2 and from *Micrococcus* strain I 3 (18) were included as references.

Haemagglutination. Normal and tanned sheep erythrocytes were sensitized with saline extracts of the bacteria and agglutinated in *Staph aureus* Cowan I immune serum which contains antibodies against *Staph aureus* sensitizing antigens (17).

RESULTS

Agglutination

Agglutinins in rabbit preimmune sera. A number of rabbit preimmune sera were examined for the presence of agglutinins against micrococci. Table I illustrates the reactions in 3 sera. The majority of the *Micrococcus* strains agglutinated strongly in undiluted preimmune sera. Most were negative in 1/10 dilution but some reactions were observed up to 1/40–1/80. *Staph aureus* and *Staph epidermidis* gave similar results.

TABLE I
Agglutination in Rabbit Preimmune Sera

No of strains tested	Serum I				Serum II				Serum III			
	1	1/10	1/20	1/40	1/1	1/10	1/20	1/40	1/1	1/10	1/20	1/40
	—	—	—	—	—	—	—	—	—	—	—	—
<i>Micrococcus</i>	19	15	1	0	16	7	9	1	1	11	1	0
<i>Staph aureus</i>	19	11	4	0	14	4	2	1	8	11	0	0
<i>Staph epidermidis</i>	8	7	3	0	5	1	0	0		0	0	0

TABLE 2
Absorption of Rabbit Preimmune Serum II

Strains agglutinate 1	Serum titres				
	Before absorption	<i>M luteus</i> 810	<i>M conglom</i> 84	<i>Staph aur</i> 830	<i>Staph epif</i> 1677
<i>M luteus</i>	810	80		40	1
<i>M conglom</i>	84	10	10	~	
<i>Staph aur</i>	830	40	40	90	10
<i>Staph epif</i>	1677	20	20	1	~

Reciprocal values

means = agglutination in undiluted serum

Absorptions were carried out to see if all reactions were due to one and the same antibody. Table 2 shows that this cannot be the case. The agglutinations observed in preimmune sera are produced by more than one antibody, probably several some of which are apparently common to micrococci and staphylococci whereas others are more specific. A micrococcal immune serum which did not agglutinate the homologous strain prior to immunization may therefore nevertheless contain pre-immune antibodies in low titres against other micrococcal strains and against staphylococcal strains. This can hardly be avoided. Preimmune serum had not been preserved in sufficient quantities to permit control agglutinations with all strains which were positive in the corresponding immune serum. By agglutination in immune serum it was therefore difficult to evaluate the low titres which might be due either to preimmune or to immune antibodies. Titres $\geq 1/40$ in the following are estimated as significant for immune antibodies.

Immune sera against *Micrococcus*, *Staph aureus* and *Staph epidermidis* strains: cross agglutinations and cross absorptions. The *Micrococcus* strains and a number of *Staph aureus* and *Staph epidermidis* strains agglutinated in *Micrococcus*, *Staph aureus* and *Staph epidermidis* immune sera. The reaction pattern was the same as in preimmune sera, i.e. extensive cross reactions in low titres. The production by immunization of genus heterologous antibodies did not seem to be very extensive but could easily be demonstrated particularly in immune sera which had only small amounts of preimmune antibodies. Table 3 gives an example of this. Cross reacting antibodies are therefore both preimmune and immune, apparently depending upon which strains of staphylococci and micrococci the rabbits have exposed before immunization.

Successive absorptions were then carried out in 10 *Micrococcus* immune sera. The sera were agglutinated before absorption and after each absorption with the set of strains mentioned above. The absorptions confirmed that micrococci and staphylococci have more common

antigens. When micrococcal immune sera were absorbed with one or two *Staph aureus* strains agglutination regularly disappeared with all staphylococcal strains but also with the majority of *Micrococcus* strains whereas the homologous strain and usually a few other *Micrococcus* strains were still positive. Therefore in addition to the shared antibodies micrococcal immune sera also have antibodies specific to micrococci. Table 4 in which the results of absorptions of *M luteus* 133, immune serum have been compiled illustrates this. It can be deduced that serum 1335 has at least two antibodies common to micrococci and staphylococci and at least two additional antibodies apparently specific for micrococci.

TABLE 3
Agglutination in *M luteus* 1335 Immune Serum

	No of strains tested	No of strains agglutinating in Serum dilution							
		1	10	20	40	80	160	320	640
<i>Micrococcus</i>	18	13	11	8	4	2	2	1	1
<i>Staph aureus</i>	19	13	5	4	1	0	0	0	0
<i>Staph epiderm</i>	8	4	2	2	1	0	0	0	0

Reciprocal value
§ Homologous strain

TABLE 4
Absorptions of *M luteus* 1335 Immune Serum

No of strains tested	Before absorption	No of strains agglutinating in <i>M luteus</i> 1335 serum After successive absorptions with				
			<i>Staph aur</i> W 46	<i>Staph aur</i> 830	<i>M conglomer</i> 740	<i>M luteus</i> 210
<i>Micrococcus</i>	18	13	1	4	2	0
<i>Staph aureus</i>	19	13	2	0		
<i>Staph epiderm</i>	8	4	1	0		

Strong agglutination with *M luteus* 1335 and 210 weak with *M conglomer* 740 and 94
Agglutination with *M luteus* 1335 and 210

The agglutinating and absorbing abilities of *Staph aureus* Wood 46 the strain used for the first absorption in these experiments were not destroyed by autoclaving, or treatment with trypsin. Thus the most widely distributed of the agglutinogens common to micrococci and staphylococci do not seem to be of protein nature.

Agglutination in Staph aureus factor sera The agglutinogens common to micrococci and staphylococci have been interpreted above as group antigens. To make sure that the cross reactions were not due to the presence of specific *Staph aureus* agglutinogens in micrococci the

Micrococcus strains were agglutinated in our set of 13 *Staph aureus* factor sera. The majority of the *Micrococcus* strains agglutinated in one or two factor sera but the reactions were weak and scattered. They were interpreted as resulting from preimmune antibodies against micrococci. These antibodies had not been removed by the *Staph aureus* strains used for absorption because they lacked the corresponding antigens (see above).

Specific Micrococcus agglutinogens The presence of specific micrococcal agglutinogens and the antigenic relationship between the *Micrococcus* strains were evaluated by titrations of the agglutinins of the *Micrococcus* immune sera and by successive absorptions. After each absorption a decreasing number of *Micrococcus* strains was agglutinated and when a serum had been absorbed with 3 or 4 strains an antibody remained which in most instances agglutinated the homologous strain exclusively.

TABLE 5
Micrococcus Strains with Similar but not Identical
Patterns of Agglutinogens

Group 1	<i>M. luteus</i>	810
		210
		1335
Group 2	<i>M. roseus</i>	861
		691
	<i>M. luteus</i>	144
Group 3	<i>M. conglomer</i>	740
		84
	<i>M. varians</i>	547
Group 4	<i>M. varians</i>	984
		1146
		719

Like *Staph aureus* the micrococci seem to have a number of specific agglutinogens. The individual strains share some of these agglutinogens in different combinations and can be characterized by patterns of antigens. No agglutinogens characteristic for *Micrococcus* as a genus or for any of the four species of this material could be demonstrated. Some of the *Micrococcus* strains however were clearly related in their content of agglutinogens although none of them had exactly identical patterns. Table 5 shows groups of serologically related strains. There is a tendency towards a correlation between the species and the groups according to agglutination. But of the 12 *M. luteus* and *M. roseus* strains only 6 are found in groups 1 and 2 whereas the others have different patterns of agglutinogens.

Precipitation

Ring test precipitation was performed with extracts of the *Micrococcus* strains against some *Staph aureus* *Staph epidermidis* and *Micrococcus* immune sera. This screening gave quite distributed precipitations but not such frequent reactions as agglutination. Cross precipitations occurred between *Staph aureus* *Staph epidermidis* and micrococci. More specific reactions were not to be expected with these crude antigens.

Agar gel precipitation All but two *Micrococcus* strains produced one or more precipitation lines against the homologous immune serum. Of these all except two strains also produced lines against one or more heterologous *Micrococcus* immune sera. The following precipitation lines were identified by means of known reference systems: four strains produced a polysaccharide A line (beta glucosaminyl ribitol teichoic acid) and 3 strains an I B line (alpha glucosaminyl glycerol teichoic acid) (Table 6). The precipitation lines did not show any correlation with the species or with the results of agglutination.

TABLE 6
Agar Gel Precipitation Lines Identified

1 1 saccharide A line	<i>M. luteus</i>	84
	<i>M. varians</i>	719
	<i>Staph. epiderm.</i>	1116
1 3 teichoic acid line	<i>M. luteus</i>	144
	<i>M. roseus</i>	149
	<i>M. varians</i>	1116

The *Staph aureus* precipitinogens polysaccharide 263 (alpha glucosaminyl ribitol teichoic acid) protein A and antigen B were not demonstrated in any of the *Micrococcus* strains. Absorptions were not carried out.

Haemagglutination

Extracts of all the *Micrococcus* strains and some *Staph epidermidis* strains were tested for their ability to sensitize normal and tanned sheep erythrocytes. When the treated erythrocytes were examined in dilutions of *Staph aureus* Cowan I immune serum all *M. luteus*, *M. roseus* and *M. conglomeratus* strains gave a negative or doubtful agglutination whereas all three *M. varians* strains (the two strains (*M. conglomeratus* 157 *M. varians* 529) which were shown to belong to *Staph epidermidis* and all the other *Staph epidermidis* strains examined gave a strong haemagglutination. This indicates that *M. luteus*, *M. roseus* and *M. conglomeratus* lack the heterogenetic antigen sensitizing normal erythrocytes and the staphylococcal antigen sensitizing tanned cells whereas *M. varians* has them.

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IN VITRO STUDIES OF SOME IMMUNOLOGICAL PHENOMENA IN EXPERIMENTAL RABBIT TOXOPLASMOSIS

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It is well known that in many hosts toxoplasmosis is accompanied by the development of both circulating antibodies and delayed sensitivity. This relationship is the case in man and in a number of laboratory animals *e.g.* the rabbit. Animals which have passed an infection caused by a strain of low virulence are protected against an otherwise fatal disease if exposed to a virulent strain. On the other hand rabbits sero-positive after immunization with killed *Toxoplasma* organisms succumbed after challenge with the virulent RH strain even when antibody titres were high. These animals were toxoplasmin negative while rabbits with high post infection antibody titres were positive (16). However the relationship between the demonstrated immune reactions and protection to challenge is not clear.

Phytohaemagglutinin was first shown by Nowell (23) to induce blastogenesis in peripheral blood leukocytes *in vitro*. This observation later was confirmed and evidence presented that the blood cells affected by phytohaemagglutinin were small lymphocytes (23). Other agents such as staphylococcal filtrate and streptolysin S were reported to cause nonspecific blastogenesis (12, 20, 27, 28). Specific antigenic stimulation of lymphocytes from previously sensitized donors also has been reported for a number of antigenic substances (23), the most effective of which seems to be purified tuberculin protein derivative (PPD). Specific stimulation also can be induced with rabbit anti lymphocyte serum (8, 10, 27, 28) and with homologous lymphocytes from an unrelated donor (2, 26).

Little is known about the mechanism of blastogenesis after stimulation with these agents or of the function of the transformed cells. So far no convincing facts have been presented to prove any fundamental difference in blastogenesis after nonspecific and specific stimulation. No histologic or metabolic difference between blastoid cells formed after stimulation with the different agents have been reported. Although some researchers have attributed immunological properties

to the transformed cells (1 II 24) no other function than mitosis has been adequately proven (24) —However it has been claimed that the *in vitro* formed blastoid cells closely resemble the large pyroninophilic cells found *in vivo* in homografts and graft versus host reactions and also the blast cells found in lymphatic tissues after antigenic stimulation (24)

Preliminary investigations showed that both toxoplasmin and living *Toxoplasma* can stimulate transformation of sensitized rabbit lymphocytes. Therefore a comparison of the ratio of blastoid cells after stimulation of peripheral blood lymph node and spleen lymphocytes might give an idea of the existence and distribution of sensitized immunocompetent cells.

The capacity of the *Toxoplasma* organisms to invade cells and multiply in the various cultures used was studied. An attempt was made to inhibit the infection of lymphoid cell cultures with high titre antisera from rabbits immunized with live or killed *Toxoplasma* organisms.

MATERIAL

Rabbits Twenty four young healthy toxoplasmin and sero negative (dye test complement fixation test) rabbits weighing around 25 kg were used. The rabbits were kept as described earlier (14).

***Toxoplasma* strains** Sabin's strain RH was used throughout for the production of antigen for the dye and complement fixation test and for toxoplasmin. The same strain was used as a stimulant and infective agent for lymphocyte cultures.

A non fatal infection of rabbits was obtained with the strain toxo 676 67^m. The strain was originally isolated from a human case and obtained through the courtesy of Dr J Chr Sum of the State Serum Institute of Copenhagen.

Toxoplasmin For stimulation of lymphocytes a purified preparation of strain RH was used. Toxoplasmin prepared as described previously (16) was passed through a Sephadex C 200 column in 0.1 M Tris-HCl 0.5 M NaCl 2 per cent butanol buffer pH 8.0. *Toxoplasma* specific antigenic activity was demonstrated only in the high molecular peak and the antigen could thus be separated from the main part of extraneous protein.

The crude preparation together with the control antigen described earlier (16) were used for skin tests.

Von Specific stimulant 1 hytohaemagglutinin (PHA) · Wellcome batch h639 was used.

METHODS

Immunization of rabbits Twelve rabbits were inoculated in the foot pad with 0.5 ml of a suspension of brains from 5 mice infected with the toxo 676 67^m strain. After 3-4 weeks sera were positive to high titres in both dye test and complement fixation test, however the toxoplasmin reaction was not positive until 5-8 weeks after inoculation. Five rabbits were immunized with killed *Toxoplasma* organisms as described in a previous paper (15).

Serological examination Dye test (DT) and complement fixation test (CIT) were performed as described earlier (13).

Lymphocyte Suspensions

Peripheral blood Blood was obtained by ear laceration and defibrinated with glass beads. Gelatin fractionation of the cell population was carried out according to the procedure described by Coulson & Chalmers (3). The cells were washed once in Earle's medium 199 containing 300 IU penicillin and 100 gamma strep-

tomycin per ml and resuspended. The concentration of lymphocytes was determined with a haemocytometer and their viability tested with trypan blue. The suspensions were adjusted to 2×10^6 lymphocytes per ml. Less than 1 per cent of the lymphocytes were non viable.

These suspensions always contained erythrocytes in about the same concentration as leukocytes. Of the leukocytes 90-95 per cent were lymphocytes, 1-2 per cent were monocytes and the remaining cells were granulocytes.

Lymph node and spleen. The lymph node cell suspension was obtained from the two popliteal lymph nodes. Lymph node or spleen was cut into small pieces and passed through a Borel sieve into 5-10 ml Parker's medium. After centrifugation, washing once and viability test, the concentration was adjusted to 2×10^6 viable cells per ml.

The cell suspensions from lymph nodes contained 70-80 per cent lymphocytes. Most of the remaining cells were macrophages. About 10-20 per cent of the cells, mostly macrophages and large lymphocytes, were non viable.

The spleen cell suspension contained macrophages in the same proportion as the lymph node cell suspensions. About 5-15 per cent of the cells were non viable, mostly macrophages and large lymphocytes.

Cell cultures. One ml of cell suspension was added to a 10×80 mm tube containing 0.3 ml fetal calf serum. To this mixture was added 1 ml Parker's medium containing either PHA, toxoplasmin or living *Toxoplasma*.

The cultures were observed for 96 hours. Incubated at 37°C, the number of live lymphocytes decreased with 10 per cent or less within this time.

Cell preparations for microscopical examination. The apparatus described by Dore & Balfour (5) was used. Of the cell suspension 0.1 ml was taken from the bottom of a tube. The cells were sedimented and flattened on a small area of a glass slide by centrifugal force. Simultaneously the fluid of the suspension was drained by a filter paper surrounding this area. This method gave good preparations with easily observed details in the separate cells. The cell preparations were fixed in methanol and stained by the May-Grunwald-Giemsa methods. Five hundred lymphocytes were counted in each preparation.

Toxoplasma suspensions. Infected mouse peritoneal exudate contained free *Toxoplasma* organisms and a considerable number of macrophages, many of which contained multiplying parasites. A pure suspension of free parasites was obtained by the following procedure. One-2 ml freshly harvested mouse peritoneal exudate was diluted with 6-8 ml Parker's medium and centrifuged for 15 minutes at 2000 g . The supernatant was discarded and the cells resuspended in 6-8 ml Parker's medium. The suspension was filtered through a millipore filter, pore size 8μ , with a positive air pressure of 15 kg/cm². No cells passed the filter, however, by turning the filter upside down the *Toxoplasma* organisms could be flushed out of the filter, leaving the macrophages firmly attached. By this technique a pure *Toxoplasma* suspension containing about 20 per cent of the original number of organisms was obtained. Viability tests showed that 7-10 per cent of the *toxoplasms* were dead after the procedure compared to 3-5 per cent in the untreated exudate.

EXPERIMENTAL AND RESULTS

Lymphocyte cultures were investigated from three groups of rabbits, namely:

- 1) Toxoplasmin and sero negative (10 animals)
- 2) Toxoplasmin negative but sero positive in high titre ($DT \geq 1/1250$, $CFT \geq 1/120$) after immunization with killed *Toxoplasma* organisms (10 animals)
- 3) Toxoplasmin and sero positive ($DT \geq 1/1250$, $CFT \geq 1/120$) after infection with the toxo 626/62 strain (10 animals)

Five rabbits were inoculated 6 months before the experiment and five only 7-9 weeks prior to the investigation when the toxoplasmin

reaction had become recently positive. There was no principal difference in the reactions recorded in these two subgroups. Therefore they were pooled for the transformation experiments whereas they are accounted for separately in the section on susceptibility to *Toxoplasma* infection for reasons that will be given later.

Transformation ratio. The following stimulants were added to the cultures:

- 1) PHA in a final dilution of 1/100
- 2) The purified toxoplasma preparation in a final dilution of 1/100 (16 experiments). Preliminary investigations had shown that this dilution gave optimal lymphocytic stimulating activity. —The crude preparation in a final dilution of 1/10 (4 experiments). When used in these dilutions parallel tests showed almost identical transformation ratios for the two preparations.
- 3) *Toxoplasma* organisms suspended in Parker's medium in four concentrations corresponding to doses of 10^2 , 10^3 , 10^4 and 10^5 parasites per 10^6 lymphocytes. It was found that a dose of 10^3 was most suitable for studies of transformation although some parasite multiplication and release of antigen occurred. Higher parasite concentrations often caused vigorous infection of the cultures leading to cell degeneration after 2 or 3 days and the lower concentration often seemed to have too weak an antigenic effect.

In each set a control tube was included containing only lymphocytes serum and Parker's medium.

On account of the limited number of cells that were obtained from each animal only two sets of cultures could be prepared. Thus each tube had to serve for two consecutive samples (on days 1 and 2 or 3 and 4 respectively).

Cells were classified as transformed when showing marked enlargement, diameter ≥ 12 , pronounced basophilia of the cytoplasm and a light slightly acidophilic area close to the nucleus. The nuclear chromatin also had to be dispersed and the nucleoli distinct. These cells are morphologically similar to the haemocytoblast as described by Faagruus (7). Cells showing doubtful transformation were not included.

Besides the transformation of lymphoid cells typical changes were seen in macrophages in cultures with multiplying *Toxoplasma*. Beginning 24 or 48 hours after inoculation the macrophages showed increased marginal basophilia of the cytoplasm and mitosis in ≥ 3 per cent of the cells. Such cells resembled the blastoid cells but were easily distinguishable particularly because of the typically shaped nucleus with coarse chromatin.

Transformations were seen after 24 hours and their incidence increased rapidly thereafter. This change occurred both after stimulation with PHA and after antigenic stimulation. Generally mitoses were not observed before 48 hours. They were seen almost invariably in cul-

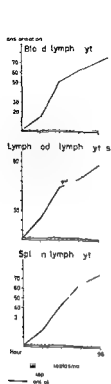


Fig 1

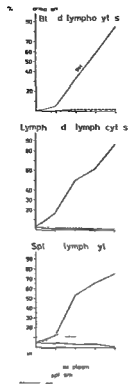


Fig 2

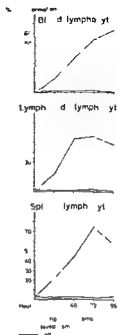


Fig 3

- Fig 1** Transformation ratio in lymphocyte cultures from toxoplasmin and sero negative rabbit. Mean percentage of blast forms from 4 readings during the observation period. Measurements on day 1 performed immediately before the addition of stimulant to the culture.
- Fig 2** Transformation ratio in lymphocyte cultures from toxoplasmin negative but sero positive rabbits. Mean percentage of blast forms from 4 readings during the observation period. Measurements on day 1 performed immediately before the addition of stimulant to the culture.
- Fig 3** Transformation ratio in lymphocyte cultures from toxoplasmin and sero positive rabbits. Mean percentage of blast forms from 4 readings during the observation period. Measurements on day 1 performed immediately before the addition of stimulant to the culture.

tures showing transformations but in small proportions corresponding to 1-5 per cent of transformed cells.

In Figs 1-3 the transformation ratio is indicated for peripheral blood lymph node and spleen lymphocytes for each group of rabbits. As could be expected the transformation ratio after PHA stimulation was very high.

The statistical significance of the increase in antigenically stimulated lymphocytes is indicated in Table 1. As can be seen a significant increase was demonstrated in lymphocytes from all three organ origins from both toxoplasmin and sero-positive rabbits. In rabbits toxoplasmin negative but sero positive the transformation ratio was signifi-

fically increased in spleen but not in lymph node lymphocytes. In peripheral blood lymphocytes blastoid cells were present in very low proportion before stimulation. During the observation period the ratio of such cells increased to values of only around 1 per cent. Although the increase was statistically significant for both stimulants the number of transformed cells was very low and can hardly reflect any specific biological reaction. In lymphocytes from toxoplasmin and sero-negative rabbits a slightly significant increase in blastoid cells was demonstrated for spleen lymphocytes after stimulation with living *Toxoplasma*. The data do not permit any explanation for this finding.

From Figs 1-3 and Table 1 can be seen that in all samples the percentage of blastogenesis was somewhat higher when living parasites were used as a stimulant instead of toxoplasmin.

Susceptibility of the Various Lymphocyte Cultures to Toxoplasma Infection

It was considered advisable to compare in the same experiment the lymphocyte cultures from rabbits belonging to the different experimental groups. For practical reasons however it was not possible to examine cultures from more than two rabbits simultaneously. The investigation was divided therefore into two main experiments each including lymphocyte cultures from five pairs of rabbits grouped as follows:

Experiment I

- 1) Toxoplasmin and sero-negative rabbits (group =)
- 2) Rabbits toxoplasmin and sero-positive after infection 6 months earlier (group ++)

Experiment II

- 1) Rabbits toxoplasmin negative but sero-positive after immunization with killed parasites (group \pm)
- 2) Rabbits toxoplasmin and sero-positive after infection 7-9 weeks earlier (group ++)

Cultures were classified as infected when multiplying *Toxoplasma* organisms were found inside one or several cells in microscopic fields of view containing a total of 500 lymphocytes on one of the first three days and the same or corresponding culture proved to be infected on the following day also.

Blood Lymphocyte Cultures

Table 2 shows the degree of infection 24 hours after inoculation. The values are present in a ratio of the number of infected cells per 1000 lymphocytes over the number of infected cultures. After 24 hours

neither ruptured host cells nor extracellular *Toxoplasma* organisms were seen and the ratio of infected to total cells will approximate therefore the proportion of *Toxoplasma* organisms in the inoculum which had penetrated into cells. Table 2 shows that infected cells were found only in cultures from toxoplasmin negative rabbits and that such cells were few in numbers.

TABLE 2

Mean Number of Infected Cells per 1000 Lymphocytes in Infected Cultures over Number of Infected Cultures at 24 Hours after Inoculation Groups of Rabbits Indicated as Follows: = Toxoplasmin—and Sero negative ++ Toxoplasmin—and Sero Positive after Infection 6 Months Earlier —+ Toxoplasmin Negative but Sero Positive after Immunization with Killed Parasites # Toxoplasmin—and Sero Positive after Infection 7-9 Weeks Earlier

Exp	Rabbit group	Blood		Lymph node		Spleen	
		No toxopl/10 ⁶ lymphoc		No toxopl/10 ⁶ lymphoc		No toxopl/10 ⁶ lymphoc	
		10	10 ⁴	10	10 ⁴	10	10 ⁴
I	= ++	5/2 0/0	2/1 0/0	12/4 7/4	5/2 0/0	6/3 5/4	6 ⁷ 3 ⁷
II	—+ #	2/1 0/0	0 0 0/0	35/5 3 1/5	14/5 11/5	39/4 20/3	10/3 5 ⁷

TABLE 3

Lowest Infective Dose Giving Demonstrable Infection at 96 Hours after Inoculation Groups of Rabbits Indicated as Follows: = Toxoplasmin—and Sero Positive after Infection 6 Months Earlier —+ Toxoplasmin Negative but Sero Positive after Immunization with Killed Parasites # Toxoplasmin—and Sero Positive after Infection 7-9 Weeks Earlier

Exp	Rabbit group	Blood			Lymph node			Spleen		
		Not inf	No toxopl/10 ⁶ lymphoc		Not inf	No toxopl/10 ⁶ lymphoc		Not inf	No toxopl/10 ⁶ lymphoc	
			10	10 ⁴		10 ³	10		10	10 ³ 10
I	= ++	1 4	1 1	3 0	0 0	3 1	2 3	0 0	1 1	1 3 0
II	—+ #	1 3	3 0	1 0	0 0	1 1	4 4	0 0	0 0	1 1 4 3

Table 3 demonstrates the lowest *Toxoplasma* concentration that gave demonstrable infection during the observation period. In the two groups of toxoplasmin positive rabbits cultures from only one of 10 animals were found infected whereas in toxoplasmin negative rabbits cultures from 11 of 10 animals were infected. Infection could be observed only with a large inoculum of *Toxoplasma* however the diffe

rence between toxoplasmin positive and negative rabbits was significant ($P < 0.001$)

The parasite multiplication and spread within the cultures was limited. Daily countings covering the entire observation period revealed only a low number of infected cells. With an infective dose of 10 parasites per 10^6 lymphocytes a total of 49 *Toxoplasma* infected cells (22 macrophages, 10 blastoid cells, 17 small lymphocytes) were found in cultures from toxoplasmin negative rabbits, whereas the corresponding figures for toxoplasmin positive rabbits was only 5 (2 macrophages, 3 small lymphocytes). In no preparation was *Toxoplasma* found within a pseudo eosinophilic leukocyte.

Lymph Node and Spleen Cultures

The data in Tables 2-3 indicate that the infection rate was far greater in cultures from these organs compared to peripheral blood. Table 2 shows that when the infective dose was 10 parasites per 10^6 lymphocytes approximately 5-39 per cent of inoculated parasites had penetrated into cells after 24 hours. A higher percentage was found with an infective dose of 10^1 organisms. The fact that in a number of cultures the calculated ratio exceeded 100 per cent might be accounted for by random variation or it might indicate that a second cycle of *Toxoplasma* growth had occurred. Table 3 shows that infection of cells also followed upon inoculation of small numbers of *Toxoplasma*.

No significant difference was demonstrated between cultures from immune and susceptible animals. The ratio of infected cells was somewhat higher throughout in experiment II than in I but this was probably due to technical factors.

In Figs. 4-6 an attempt was made to analyse further the difference in susceptibility to *Toxoplasma* infection between blood lymphocyte cultures and cultures from lymph nodes and spleen. Fig. 4 shows the distribution of different cells in the cultures immediately before inoculation with *Toxoplasma*. The blood lymphocyte cultures consisted almost entirely of small lymphocytes. Large lymphoid cells and macrophages were less than 1 per cent of the population. Pseudo eosinophilic leukocytes were found in a mean ratio of 1 per 100 lymphocytes. Cultures from lymph nodes and spleen contained macrophages in ratios of about 20 per 100 lymphocytes, as well as small lymphocytes and a small number of large lymphoid cells.

In Fig. 5 is shown the ratio of infected macrophages, blastoid cells and small lymphocytes in lymph node cultures 24 hours after infection with 10 parasites per 10^6 lymphocytes. The largest ratio of infected cells, 11-29 per cent, was found in the macrophage group. 2-10 per cent of the blastoid cells were infected, whereas infection of small lymphocytes was rare, 0.1-1 per cent. The results are representative for spleen cultures and for lymph node and spleen cultures infected

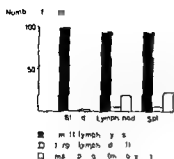


Fig 4

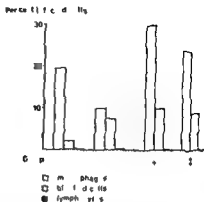


Fig 5

- Fig 4** Proportion of small lymphocytes large lymphoid cells and macrophages in blood lymph node and spleen cultures before addition of *Toxoplasma*. Data presented correspond to an average of all cultures used in the investigation.
- Fig 5** Lymph node cultures 24 hours after inoculation with 10^5 *Toxoplasma* organisms per 10^6 lymphocytes. Percentage of *Toxoplasma* infected cells in macrophages blastoid cells and small lymphocytes in the two experiments and four rabbit groups.

with 10^4 parasites per 10^6 lymphocytes. It therefore seems evident that the macrophages were of fundamental importance for the early multiplication of *Toxoplasma* in the cultures. However, it is also clear that a fairly large proportion of blastoid cells were infected (Fig 6). As the ratio of blastoid cells in several cultures especially from the spleen increased considerably during the observation period, it was presumed that the late propagation to a large extent occurred in such cells. Fig 7 presents the ratios of infected macrophages and blastoid cells from all types of cultures 48 hours after inoculation with 10^5 and 10^6 toxoplasms per 10^6 lymphocytes. At this time the number of infected cells had increased considerably, indicating secondary infection by toxoplasms released from initially infected cells. The figure suggests that the increase was larger in blastoid cells than in macrophages; however, the difference was not statistically significant. The percentage of infected small lymphocytes at this time was still ≤ 1 per cent. 48 hours after inoculation the number of intracellular parasites was very large. In many cases around 100 parasites were seen within one macrophage and 60-70 toxoplasms in one blastoid cell. In both types of cells the parasites were often located in several clones. No difference in the number of intracellular parasites was observed between cultures from immune and susceptible rabbits. More than two parasites per cell were seldom seen within small lymphocytes.

In order to investigate if the demonstrated susceptibility of blastoid cells to *Toxoplasma* infection applies to blastoid cells in general or only to antigenically stimulated transformed lymphocytes, the ratio of infected cells in blood lymphocyte cultures treated and untreated with PHA 2 days earlier were investigated. Twelve cultures from the same



Fig 6

Transformed lymph node lymphocytes containing multiplying *Toxoplasma* organisms

sero and toxoplasmin negative rabbit were inoculated 11 of which were unstimulated and 6 stimulated with PHA. At the time of inoculation the ratio of blastoid cells was 0.2 per cent in the former and 4.3 per cent in the latter group. Twenty four hours after inoculation with 10^6 *Toxoplasma* organisms per 10^6 lymphocytes the mean number of infected cells per 1000 lymphocytes was 11 ± 2.9 in the unstimulated and 121 ± 6.0 in the stimulated group. 83 per cent of the infected cells in the stimulated cultures were blastoid forms. The difference between the two groups is statistically significant $P < 0.001$. Pseudo eosinophilic leukocytes were found in a frequency of 3.4 per cent in the cultures. 11 per cent of these cells were found infected during the observation period. After 24 hours the granulocytes frequently contained multiplying *Toxoplasma* however the parasites stained poorly and were

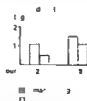


Fig 7

Percentage of infected macrophages and blastoid cells 48 hours after inoculation. The values are calculated from the cumulative data from all lymph node and spleen cultures inoculated with either 10^4 or 10^6 *Toxoplasma* organisms per 10^6 lymphocytes.

not clearly defined in the surrounding cytoplasm. From 48 hours after inoculation they were detected only with difficulty within such cells. Most probably they were being destroyed within these cells.

Inhibitory Effect of DT and CIT Positive Serum on Penetration and Multiplication of Toxoplasma

Since *Toxoplasma* multiplication was poor in blood lymphocyte cultures only lymph node and spleen cultures were investigated. Lymphocytes from two rabbits toxoplasmin and sero positive (group ++) and two toxoplasmin and sero negative (group =) were tested.

Two DT and CIT positive sera were used. One from a rabbit that had passed an infection 3 months earlier (DT 1/8750 CIT 1/1920). The second serum was pooled from two rabbits immunized with killed parasites 3 months earlier (DT 1/1950 CIT 1/240). They were stored at -20°C and used within 4 weeks after bleeding.

The cultures were set up as described earlier. Instead of fetal calf serum 0.1-0.3 ml positive rabbit serum was added to the lymphocyte suspensions. The rabbit immune serum was used: 1) native 0.3 ml (n) 2° inactivated at 36°C for 30 minutes 0.3 ml (i) 3) 0.1 ml inactivated rabbit serum and 0.2 ml human access 7 serum (a). Fetal calf serum was used as a control.

Toxoplasma suspensions were added in two concentrations 10^6 and 10^4 per 10^6 lymphocytes. Owing to the large number of tubes lymphocyte cultures from only one animal could be examined in each experiment.

All control cultures were degenerated after 96 hours. The data in Table 4 demonstrate that both sera used had a protective effect. In a few cultures, however, where inactivated serum with or without necessary factor was added this protection was overcome by the invading parasites resulting in widespread infection and cell degeneration. Such infection occurred in one of 48 cultures in group ++ and in 6 of 48 cultures in group =. In a few more cultures, mainly in group =, a small number of infected cells were found indicating an incomplete protection.

DISCUSSION

Small lymphocytes from toxoplasmin and/or sero positive rabbits were transformed into blastoid cells when stimulated with either toxoplasmin or living *Toxoplasma* organisms, whereas lymphocytes from toxoplasmin and sero negative rabbits did not show transformations to any significant degree (with the exception of spleen cells stimulated with living *Toxoplasma*). This result indicates that the transformations were the result of a specific stimulation of previously sensitized cells.

It was demonstrated that the percentage of blastoid cells in the population was larger after stimulation with living *Toxoplasma* than when toxoplasmin was used. The reason might well be that a greater number of antigenic substances was released in cultures containing living parasites.

TABLE 4

Inhibitory Effect of DT and CFT Positive Sera on Infection of Lymph Node and Spleen Cultures The Data were Obtained from Observations 96 Hours after Inoculation Serum P = Immune Serum Postinfectious I = Immune Serum after Immunization with Killed Parasites C = Control Fetal Calf Serum n = Native i = Inactivated a = Inactivated + Human Accessory Factor Number of Infected Cells per 1000 Lymphocytes/Number of Infected Cultures Deg = Cultures Degenerated by Vigorous Toxoplasma Infection

Lymph Node Lymphocytes

Group ++			Group —		
Serum	No toxoplasma/10 ⁶ lymphocytes		Serum	No toxoplasma/10 ⁶ lymphocytes	
	10 ⁻	10 ⁺		10 ⁻	10 ⁺
C	deg./2	deg./2	C	deg./2	deg./2
Pn	0	0	Pn	1/1	0
Pi	1/1	0	Pi	1/1	deg./1
				deg./1	
Pa	0	0	Pa	1/1	deg./1
				deg./1	
In	0	0	In	1/1	2/1
Ii	0	0	Ii	3/3	2/2
Ia	deg./1	0	Ia	6/2	1

Spleen Lymphocytes

Group ++			Group —		
Serum	No toxoplasma/10 ⁶ lymphocytes		Serum	No toxoplasma/10 ⁶ lymphocytes	
	10 ⁻	10 ⁺		10 ⁻	10 ⁺
C	deg./2	deg./2	C	deg./2	deg./2
Pn	0	0	Pn	0	0
Pi	1/1	0	Pi	1/1	deg./1
				deg./1	
Pa	0	0	Pa	3/3	2/1
In	0	0	In	0	0
Ii	0	0	Ii	2/2	2/1
Ia	4/1	0	Ia	31/2	0

Blastoid cells were found in considerably larger proportions in stimulated spleen cell cultures than in cultures from peripheral blood and lymph nodes. Possibly the spleen contained a higher percentage sensitized lymphocytes or the spleen lymphocytes were more reactive than lymphocytes from the other sources. It is well known (11) that intravenous injection of particulate antigens is followed by antibody production that takes place mainly in the spleen and to a smaller extent in lymph nodes. Under these conditions probably a larger number of lymphocytes are sensitized in spleen than in lymph nodes and peripheral blood. In the present experiment one group of rabbits was im-

munized intravenously with killed *Toxoplasma* while in the other group an early appearing parasitemia (14) may have played a role similar to intravenous inoculation. These conditions would favour the supposition that spleen cells were particularly sensitized. On the other hand transformation after PHA stimulation was not observed to any greater extent in spleen than in peripheral blood and lymph node lymphocytes. This observation does not support the idea that the spleen cells were more reactive than lymphocytes from blood and lymph node.

In sensitized rabbits up to 35 per cent of spleen lymphocytes were found transformed after 96 hours. It does not seem very likely that this reflects the number of lymphocytes originally sensitized. The finding might to some extent be due to selective multiplication of sensitized cells. However this phenomenon cannot entirely explain the large percentage of transformed lymphocytes. The mechanism of blastogenesis is probably complex and it might involve stimulation of non sensitized cells as has been suggested by other investigators (11-27). No conclusions about this mechanism can be drawn from the present study.

Blastogenesis was more pronounced in lymphocyte cultures from rabbits which became toxoplasmin and sero positive after infection than in cultures from rabbits sero positive only after immunization with killed parasites. In the latter group a significant increase in blastoid cells was shown only in spleen lymphocytes. The difference might be merely of a quantitative nature corresponding to a more intensive antigenic stimulation in the former group. However it is known that the percentage of lymphocytes responsible for cell bound immunity is larger in peripheral blood and lymph nodes than in the spleen (22). Blastogenesis in peripheral blood and lymph node lymphocytes from toxoplasmin positive rabbits therefore might indicate the presence of cell bound immunity.

A problem of considerable interest is the following. Do small lymphocytes sensitized against *Toxoplasma* perform an immunological function of significance for immune protection? The role of small lymphocytes in the immune response to homograft and graft against host reaction has been established and reviewed by Cowans (9). However only a few studies have been reported so far indicating that small lymphocytes produce protective immunity against infective agents. Kempe (18) reported a case of progressive vaccinia in a child with normal gamma globulin. Massive doses of immune gamma globulin had no effect of the lesions but their progress was halted dramatically by injection of leukocytes from recently vaccinated donors in the neighboring skin. Larsh *et al* (19) showed that peritoneal exudate cells obtained from a donor mouse infected with *Trichinella spiralis* caused the accelerated removal of adult worms when injected into a recipient. Recently Dineen & Wagland (4) showed that cells obtained from mesenteric lymph nodes of inbred guinea pigs infected with *Trichostrongylus colubriformis* caused resistance when injected within eight

days before challenge inoculation. The transfer of massive volumes of serum from resistant to susceptible animals was without effect.

The present investigation does not give unequivocal evidence that sensitized small lymphocytes are involved in the mechanism of immune protection in toxoplasmosis. The observed difference in the proportion of infected blood lymphocyte cultures obtained from toxoplasmin positive or negative animals was statistically significant but as the total number of infected cells was small the biological significance of this observation remains to be clarified.

The data suggest that blastoid cells are more easily infected with *Toxoplasma* than untransformed lymphocytes. During the entire observation period only 1 per cent or less of the small lymphocytes were found infected and 24 and 48 hours after inoculation 8 per cent and 14 per cent respectively of the blastoid cells were infected. One possible reason for the greater susceptibility of blastoid cells might be an altered cell surface structure permitting easier penetration for the parasites. The fact that blastoid cells are readily infected and support vigorous multiplication of the *Toxoplasma* organisms strongly suggests that in this stage of development the cells have no immune protective function.

Of certain immunological interest are the macrophage cells present in the cultures. It was demonstrated by *Vischer & Suter* (29) in experiments with cultures of peritoneal macrophages from laboratory rodents that *Toxoplasma* can be propagated successfully in such cultures and that the intracellular multiplication of the parasites was inhibited in macrophages from rats or guinea pigs actively immunized with living *Toxoplasma*. The results of the present investigation are in agreement with the first observation. In lymph node and spleen cultures a larger number of macrophages were found infected than would be expected from their distribution and their proportional mass. This may be a result of the phagocytotic activity of the macrophages.

The second observation by *Vischer & Suter* could not be confirmed. No significant difference in the percentage of infected macrophages or in the number of intracellular parasites was found between cultures from immune and susceptible animals. Despite the fact that several authors have presented evidence to show that macrophages from immune animals have a specific ability to inactivate and destroy intracellular infective agents, other researchers have not been able to demonstrate the same phenomenon (24). The importance of macrophages in the immune mechanism is still obscure.

One cell type had an unequivocal destructive effect on *Toxoplasma*, namely the granulocyte. The rapid intracellular killing of the parasite by such cells has been demonstrated previously (15) and in this investigation. This phenomenon which evidently is non-specific in nature may be due to the action of proteolytic enzymes present in high concentration in the granules of these cells.

Lycke et al (21) have demonstrated that the penetration of *Toxoplasma* into cells *in vitro* could be inhibited by a mixture of immune serum and accessory factor as well as by the same mixture when the heat labile components had been destroyed. The results of the present investigation are consistent with these findings. Inhibition of *Toxoplasma* infection in lymph node and spleen cultures could be demonstrated both with native and inactivated DT and CFT positive serum.

The present investigation shows that DT and CFT positive serum from rabbits immunized with killed parasites had as effective an inhibitory effect as serum from a rabbit sero positive after an active toxoplasmic infection. This finding suggests that the difference in susceptibility to challenge *in vivo* between rabbits immunized with killed *Toxoplasma* and rabbits with high post infection antibody titres is not due to any fundamental difference in the antibody response against living and killed parasites.

A difference in the protective effect of DT and CFT positive serum between cultures from immune and susceptible animals was observed but the present limited investigation does not allow any further conclusions.

SUMMARY

The transformation to blastoid cells in lymphocyte cultures from peripheral blood, lymph nodes and spleen from rabbits with varying degrees of immunity were investigated after stimulation with toxoplasmin or living *Toxoplasma* organisms. The capacity of *Toxoplasma* to invade and multiply within these cells was studied also. Finally the inhibitory effect of DT and CFT positive sera was tested in cultures of lymph nodes and spleen.

A significant increase in blastogenesis during the observation period (96 hours) was demonstrated in all three types of lymphocyte cultures studied from rabbits toxoplasmin and sero positive after a passed infection. Lymphocyte cultures from rabbits toxoplasmin negative but sero positive after immunization with killed parasites showed a significant increase in transformations only in spleen lymphocytes. In cultures from toxoplasmin and sero negative rabbits a significant increase in blastogenesis was not observed with the exception of a minimal although significant increase in spleen lymphocytes stimulated with living parasites. The percentage of transformations was larger after stimulation with living parasites than when toxoplasmin was used.

A difference in the proportion of infected blood lymphocyte cultures from susceptible and immune rabbits observed suggests but does not prove the existence of an immune protective property of lymphocytes.

After transformation the blastoid cells were infected more easily by *Toxoplasma* and multiplication was vigorous within such cells.

It was demonstrated that macrophages present to around 20 per cent

in lymph node and spleen cultures were easily infected with *Toxoplasma* and that multiplication was pronounced within them. No difference either in the percentage of infected cells or in the number of intracellular parasites was observed between macrophages from immune and susceptible animals.

Toxoplasma organisms were rapidly destroyed within granulocytes. This activity of the host cell is probably nonspecific in nature.

In vitro infection with *Toxoplasma* was inhibited with S serum with high post infection antibody titres and with γ serum with high DT and CFT titres after immunization with killed parasites.

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TRANSACTIONS OF THE SWEDISH PATHOLOGICAL SOCIETY

Meeting December 2-3 1966

G M Berger & J G Norlen A SEMINAR ON TUMOURS OF THE URINARY BLADDER

Fifty-one Swedish pathologists representing almost all of the departments of pathology of the country had digagnosed 13 slides. Opinions on cytology were fairly uniform but conclusions with regard to degree of malignancy and infiltration differed widely. The differences were more pronounced between than within the departments though an individual usually had a constant opinion in relation to the others.

The conclusions were mostly based upon specified criteria but 5 different criteria systems had been used and this was probably in principle the cause of the differences in conclusions.

Thirteen of the 13 slides were from patients who at follow up showed invasive cancer and the two others had extensive mucosal tumour growth and clinically malignant course. Of the 165 submitted diagnoses 935 were benign 74 strong suspicion of malignancy and 456 definite cancer.

Gret Östberg H Hedeland & B Holmfelt ON THE OCCURRENCE OF ADRENAL (CORTICAL) ADENOMA ESSENTIAL HYPERTENSION AND CHANGES IN CLY- LOSE BALANCE IN AN AUTOLYSED MATERIAL

It has been suggested that about 20 per cent of the cases with so-called benign essential hypertension might have an adrenal tumour producing a adrenal cortical adenoma. Primary aldosteronism might also have a relation to lowered glucose tolerance.

All the subjects aged 20 and above autopsied at the Institute of Pathology in Malmö during 6 months (73 cases) were studied for clinical diagnosis of hypertension and glycosuria (diabetes mellitus) and autopsically found of adrenocortical adenoma. More adenomas were found in male hypertensive patients than in normotensives 189 and 84 per cent respectively ($0.01 < p < 0.05$). The incidence was identical in women with and without hypertension. The frequency of adenomas was a little higher in diabetics than in non-diabetics with hypertension, respectively ($0.1 < p < 0.05$). But this difference was not of statistical significance.

According to this study the frequency of adrenal adenomas in hypertensives with diabetes was 1.1 per cent, frequency previously published 1.1 per cent. The frequency of adenomas however is rather high 10.1 per cent in the men and 1.6 per cent in the women.

J Wajsbom & A Moberg A. A. Stina Sjöström MULTI IDIOPATHIC PANCREA- TITIS—A CLINICAL AND HISTOLOGICAL STUDY

I D Schnörer HISTOPATHOLOGICAL CHANGES IN TANDERIL INDUCED PAROTITIS

In 1962 five patients were observed in whom fever, enlarged salivary glands and a feeling of dryness in the eyes and the mouth developed after treatment with Tanderil (oxifenbutazon).

A woman of 66 who for 10 days had consumed in all 30 tablets Tanderil (3 grams) ran a fever and the parotid glands on both sides swelled. Hialographic examination did not reveal anything abnormal. A biopsy about 14 days after she had been taken ill showed periductal inflammatory infiltration composed predominantly of lymphocytes with occasional reticulocytes forming small germinal centres. The acinic part of the gland showed only a moderately pronounced oedema.

II Anulson Allan Inderquist & Anders Inderquist DIFFERENTIAL DIAGNOSIS BETWEEN ULCERATIVE COLITIS AND GRANULOMATOUS ENTEROCOLITIS (CROHN'S DISEASE) PATHOLOGICAL AND ANGIOGRAPHICAL ASPECTS

In a small number of cases the superior and/or inferior mesenteric artery had been studied preoperatively by angiography. In all the cases of Crohn's disease obliterative vascular changes in the small and medium sized intestinal vessels were observed but were found only occasionally in ulcerative colitis.

The vascular changes were regarded as non specific but as they were always found in Crohn's disease they were thought to be of diagnostic importance and an indication of a chronic disease. The angiographic picture in advanced stages of Crohn's disease differed from that in ulcerative colitis in that the former exhibited irregularities in the distribution and the calibre of the arteries, obliterations of the vessels and reduced venous return. In addition to its value as a means by which to make a differential diagnosis possible between ulcerative colitis and Crohn's disease it permits an earlier and more precise localization of the extent of the disease than otherwise obtainable by the usual barium contrast method.

I Hultquist & O Björk OBSERVATIONS IN BIOPSIES OF THE MYOCARDIUM IN CASES UNDERGOING OPEN HEART SURGERY

From cases with congenital heart disease or acquired valvular disease biopsies of the myocardium were taken with a hollow sharpened drill during open heart surgery before and after perfusion. The accordance of the 86 double biopsies which were studied by light microscopy was found to be great concerning diffuse changes such as hypertrophy but only about 50 per cent in cases of focal lesions such as scar tissue. As to interstitial oedema it was more often increased after than before perfusion which indicates that oedema in some cases might be an effect of the perfusion.

Eighteen cases were studied with electron microscopy and enzyme histochemistry. In 5 cases slight ultrastructural changes consisting of glycogen depletion and oedema of the sarcoplasm were observed. In another 3 cases changes were more severe, including mitochondrial abnormalities such as swelling, loss of cristae and matrices, marginalization of nuclear chromatin, swelling and vacuolization of sarcoplasmic reticulum and slight myofibrillar changes. For dating these changes staining for phosphorilase, succinodehydrogenase and cytochrome oxidase were of value. In one case with severe changes it was thus made probable that they had started before the perfusion.

The slight changes appeared in 3 cases of congenital heart abnormalities and 2 of acquired aortic valvular lesions whereas the severe changes were found in 3 cases of acquired aortic valvular lesions. Among the 10 cases without ultrastructural changes 7 were congenital and 3 acquired heart lesions.

J. Pantén LYMPHOBLASTOID TRANSFORMATION IN CULTURES OF HUMAN MALIGNANT LYMPHOMAS

Lymphoid tissue from 6 cases of malignant lymphoma and 8 non malignant lymph nodes were grown in a modified grid monolayer culture. The control tissue after a preliminary phase of release of preformed lymphoid cells gave rise to regular monolayers of fibroblastoid morphology. They could be subcultivated throughout about 10 passages before reaching a terminal degenerative stage. The grids could be transferred 3-5 times before losing their capacity to produce fibroblastoid cells.

The six malignant tissue specimens ceased to produce lymphoid cells after the same length of time as the controls and also formed fibroblastoid monolayers. The monolayers derived from the first 2 grid transfer generations did not differ from the control monolayers. Monolayers generated from later grid transfer generations displayed a pathological irregular growth pattern. In 3 cases reticulated elements appeared which differentiated into lymphoid cells 8-19 weeks after initiation of the cultures. The reticulolymphoid cells could not be maintained in the absence of fibroblastoid cells but cocultivation with normal fibroblasts restored the capacity for survival of the reticulolymphoid cells. Chromosome analysis of one transformed strain showed 47 chromosomes interpreted as a trisomy of no 11 or 12.

The findings suggest a specific correlation between irregular growth and lymphoblastoid transformation on one hand and malignant lymphoma on the other. The correlation cannot be considered definite however because of the small number of controls and the difficulty in growing non malignant lymphoid tissue during a sufficiently long time. It is not excluded that normal lymphnodes would show the same alterations if carried through as many grid transfers as the malignant tissue.

P. Riberfeld, C. Holm & J. Ericsson POLYSOME FORMATION AND MICROINO CYTOSIS IN *IN VITRO* TRANSFORMED HUMAN LYMPHOCYTES

The light and electron microscopic appearance of human lymphocytes isolated from peripheral blood and cultured *in vitro* in the presence of phytohaemagglutinin (PHA), staphylococcal extract (STE) and tuberculin (PPD) was investigated.

Two hours after addition of PHA electron microscopy showed some small lymphocytes containing cytoplasmic ribosomes aggregated to typical poly(rib)ome. The amount of polysomes and the number of cells with these elements increased with time. After 24 hours of PHA stimulation most cells contained numerous poly(rib)omes. Counting of ribosomes showed a greater variation of ribosomal concentration in transformed cells (700-1000) than in unstimulated cells (30-450). No clear changes (enlargement, altered interrelationships of granular fibillar and fibrinous material) were noted in some cells within 24 hours after PHA stimulation.

In transformed cells acid phosphatase activity—demonstrated by incubation in a Gomori type medium—was found in the Golgi area and in foot processes and was confined to single membrane limited bodies (lysosomes and occasional Golgi elements). Untransformed lymphocytes showed less or no activity at all. During mitosis "centrifugal" redistribution of lysosome like bodies was observed.

Ferritin molecules introduced in the cultivation medium were absorbed by micro

pinocytosis and were segregated in acid phosphatase positive bodies mainly multivesicular bodies. Micropinocytosis was greater in stimulated than in unstimulated cells.

Lymphocytes from a Mantoux positive donor transformed by LHA-STF and PPD in parallel experiments showed essentially similar ultrastructural features. However the transformation time was longer and the rate of transformation lower with PPD than with STF and LHA.

S. Lindgren CONGENITAL PRIMARY ADRENAL HYPOPLASIA

At gross examination post mortem of a boy born at term who died from a piration during delivery, absence of the adrenals was observed. Histological examination of serial sections of retroperitoneal tissue however revealed a very small adrenal. The permanent cortex was very thin on the left side and almost absent on the right side. The foetal cortex was predominant and consisted of irregularly arranged eosinophilic cells and numerous giant cells.

The case was diagnosed as congenital primary hypoplasia of the extomegalic type which should be distinguished from secondary hypoplasia in adrenocortical failure. Eleven such cases were reported in the literature among them three pairs of brothers. The condition therefore appears to be a hereditary anomaly which soon gives symptoms of cortical insufficiency. Two of the 11 patients aged 1 and 3½ years respectively are receiving substitution therapy and are alive.

I. Mikulowski SPERMATOCYTIC SEMINOMA

Up to now 32 cases of spermatocytic seminoma have been described. Differences from usual seminomas include the high mean age of the patients (57 years), the tumours have been found only in the testis and always in a pure form, absence of lymphocytic infiltration in the stroma and granulomatous reaction is not present.

In a study of 94 testicular tumours of which 84 primarily had been classified as seminomas and 10 as sarcomas, 7 (7.4 per cent) cases of spermatocytic seminomas were found. In spite of tumour invasion into large blood vessels the prognosis seems to be relatively good. Of the 7 cases 4 are living and well, 2 of the cases have been observed for 5 and 4 years respectively after operation. Two died from metastases after 1.5 and 4 years duration of disease respectively. All the cases had been operated and had received X-ray therapy postoperatively.

In 22 cases of ovarian dysgerminoma no differentiation in the direction of spermatocytic seminoma was found.

Germ cell tumours with undifferentiated cells should be called gonodysmatur germioma. The term seminoma (with the possible addition of spermatocytic) should be reserved for tumours of cells of differentiated male type.

Ingemar Hagerstrand & B. Billman ASBESTOS AND MESOTHELIOMAS

In 1907 the first case of pleural mesothelioma due to inhalation of asbestos fibres was reported. During the last 30 years many reports have appeared about the high incidence of pleural mesotheliomas in asbestos workers and since 1960 the interest has been focused on the possible relation between a latent exposure and malignant mesotheliomas of pleura and peritoneum.

The results of the examination of 33 mesotheliomas listed in the files of a relation ship between asbestos exposure and mesothelioma. The problem must still be considered unsettled.

Th Berge & T Sallén METASTATIC CARCINOMA IN THE CIRRHOTIC LIVER

Among 3642 extrahepatic carcinomas portal cirrhosis was found incidentally in 108.578 per cent of the carcinomas in the cirrhotic and 69.9 per cent in the non-cirrhotic group had metastasized among these 40.4 per cent and 51.6 per cent respectively had metastases in the liver. This difference which is not statistically significant, could be explained by the fact that most of the tumours in the cirrhotic group were in an early stage of dissemination and had metastases only in a few organs. Among the cases with metastases to more than four organs all the tumours in the cirrhotic group had metastasized to the liver but the frequency in the non-cirrhotic group was about 90 per cent. When attention was paid to the haemodynamic possibilities of tumours in different locations and the stage of dissemination the cirrhotic liver was found to be just as good a soil (probably better) for metastasis as the normal liver.

Eva Brehmer Andersson & L. Brunk TAPE STRIPPING METHOD FOR CYTOLOGICAL DIAGNOSIS OF MYCOSIS FUNGOIDES

A piece of ordinary transparent tape was applied to the affected skin area and then stripped off. This was repeated using fresh tape each time until punctate oozing appeared. A slide was held against the oozing skin for about 20 seconds. To obtain material from varying depths of the lesion stripping was continued and smears taken after every 10 tapings. The material was fixed in 95 per cent alcohol and stained with May-Grünwald haematoxylin and eosin. The smears were always taken from lesions which were covered by a macroscopically intact epidermis.

The tape method was carried out on 7 patients with clinically diagnosed mycosis fungoides and the results from 5 of the cases were presented.

The imprint smear contained numerous well preserved cells permitting analysis of the configuration of the nuclei and the distribution of the chromatin. In all cases the smears showed distinctly atypical cells giving a definite diagnosis of malignancy. In two of these cases repeated biopsies had only given a preliminary diagnosis of malignant disease.

V. Nasell ABNORMAL COLUMNAR CELL FINDINGS IN BRONCHOPULMONARY CARCINOMA

According to *Papanicolaou* cytological examination (CPE) means degeneration and destruction of large numbers of bronchial columnar cells. *Papanicolaou* found an increased CPE incidence in lung cancer.

In our sputum cytologic studies, which are characteristic of the cytological changes of the columnar cells were found as well as the cytological changes. All these abnormal columnar cell findings were called ACCF. In the cytologic studies of the material in the present study, mucous and cellular surface cells were found in other than small rounded cells. These cells probably respond to the ACCF. In different material of the cytological columnar cell alterations were found. The cytological process is a slow epithelium without cilia which is not resistant to exogenous factors.

The cytologic studies of the bronchial epithelium as well as the cytological changes in the bronchial epithelium as well as the cytological changes in the bronchial epithelium without cilia which are not resistant to exogenous factors.

surface cell expulsion becomes the starting point of the metaplastic process which in turn shows a close association with bronchial carcinoma

S Dahlgren ASPIRATION BIOPSY OF INTRA PULMONARY HAMARTOMAS

P S Persson O Öbrant & C Åhren CYTOLOGICAL AND HISTOLOGICAL EXAMINATION OF THE TESTIS IN AZOOSPERMIA

D Schiffer (Turin Italy) HISTOENZYMOTOLOGY OF NORMAL AND PATHOLOGICAL GLIA

B Lagerlöf CHROMOSOME PATTERN AND BIOLOGICAL PROPERTIES OF THE CELLS OF THE THYMUS DURING IMMUNOCYTOGENESIS

A Norrby F Knutson & P U Lunden CELLULAR DISSOCIATION IN ENZYMATICALLY PRODUCED TUMOUR CELL SUSPENSIONS

Cell suspensions produced enzymatically using trypsin in combination with DNase have high initial cellular viability and are generally considered fully dissociated. In experiments on tumour kinetics a complete dissociation is desirable as hypotheses have been presented indicating aggregation or close contact between tumour cells as a prerequisite for malignant proliferation in vivo. In the exception of certain tumour cells in advanced progression however, in enzymatic suspensions from an experimental mouse sarcoma two independent phenomena may appear resulting in clumping or aggregation of cells during the final resuspension. One is the formation of a DNase sensitive gel in which cells are entrapped. The other phenomenon is a spontaneous instant aggregation of cells in synthetic media. This aggregation was interpreted as a type of primary aggregation described by Moscona.

To achieve complete degradation of the extracellular DNase sensitive gel (probably DNP gel) in suspensions rich in cells several times the concentration of DNase recommended for enzymatic techniques was needed. The amount of DNase required probably varies from tumour to tumour.

The instant spontaneous aggregation in synthetic media was inhibited on a large scale. Aggregates were dissociated by sera from various species and murine cell free ascorbic fluid.

F Ehlén & Gustafson B Josefsson & Å C Paul ULTRASTRUCTURAL LOCALIZATION OF HEAVY METALS IN EOSINOPHILIC GRANULOCYTES

Using a previously described modification of the sulphide silver method for ultrastructural studies (Ehlén & Falkmer 1967) heavy metals were found in the specific granules from rat and cattle eosinophilic granulocytes. All other organelles of both types of eosinophilic granulocytes gave a negative reaction. The characteristic central crystalline structures of the eosinophilic granules also appeared to be devoid of heavy metals.

As the modified sulphide silver method did not permit any differentiation between zinc, iron and copper a granule fraction was isolated from cattle blood using glassy carbon distilled water for haemolysis and fractionated centrifugation (Josefsson & Paul to be published). Ultrastructurally the granule fraction was more than 95 per cent pure with well preserved granules. Atomic absorption spectroscopy revealed

3×10^{16} zinc per granulum corresponding to 20 millions of atoms of zinc. No copper or iron was found by this technique.

Cattle eosinophilic granulocytes thus contain substantial quantities of zinc localized to specific granules. Whether the metal is referable to metal-dependent enzymes or has some yet unknown function remains to be investigated.

L Grmelius A NEW ARGYRAPHILIC STAINING TECHNIQUE FOR IDENTIFICATION OF THE α CELLS IN THE ISLES OF THE HUMAN PANCREAS

U Brunk & G Skold THE OXIDATION PROBLEM IN THE SULPHIDE SILVER METHOD FOR HISTOCHEMICAL DEMONSTRATION OF METALS

The metal content and its distribution in adrenal and liver tissue as shown with the sulphide silver method differs very much according to whether paraffin embedded tissue or cryostat sections are used. This can be shown to depend on oxidation probably to sulphates, sulphates, hydroxides and possible oxides of some of the metal sulphides during the process of dehydration and paraffin embedding. The oxidation products cannot act as germs during the development. The sulphites and sulphates are later dissolved during staining. By treating the sections with hydrogen sulphide gas before mounting and deparaffinisation most of the activity can be restored but some diffusion artefacts cannot be prevented. Because of this the use of cryostat sections is recommended. Paraffin sections can be used only in cases dealing with metals the sulphides of which are resistant to oxidation.

C Moberger HISTOCHEMICAL SULPHOHYDROL REACTIONS IN STUDIES OF CELL GROWTH AND CELL RIPENING

J Ericsson, P Biberfeld & G Hilm ELECTRON MICROSCOPIC STUDIES OF EXPERIMENTAL "AUTOIMMUNE" NEPHROSIS

A nephrotic syndrome was induced in randomly bred female Sprague Dawley rats by 7-8 intraperitoneal injections of homologous kidney extract and Freund's adjuvant. Portions of the renal cortex were fixed in OsO_4 and glutaraldehyde and the tissues were conventionally processed for light and electron microscopic studies. Approximately 80 μ thick frozen sections of glutaraldehyde fixed tissues were incubated for the demonstration of acid phosphatase (AcPase) and were subsequently immersed in ammonium sulphide.

Light microscopy of paraffin sections (aldehyde fixed tissue) revealed minor alterations in the renal cortex (possible slight thickening of glomerular capillary walls, "hyaline droplets" in proximal tubules and occasional protein casts with dilatation of some portions of the nephron). Thin (approximately 1 μ) thick sections of Epon embedded glutaraldehyde fixed material incubated for the demonstration of AcPase showed reaction product in many droplets—up to 4 μ in diameter—in the proximal convoluted tubules, other droplets—stainable with toluidine blue—did not acquire reaction product despite prolonged incubation.

Electron microscopy revealed lumpy bumps (deposits on the epithelial side of the glomerular basement membrane (between the basement proper and the epithelial foot processes)) and hypertrophy of the epithelial cells. The cytoplasm of proximal tubule cells contained numerous electron dense "absorption droplets" and greatly

increased numbers of large "cytosteresomes" and cytosomes. The latter appeared to carry acid phosphatase.

The observations indicate a focal damage to proximal tubule cells manifested by increased autophagocytosis. The localization of the deposits in the glomeruli on the epithelial side of the basement membrane may signify a non glomerular origin of the antigen(s) responsible for the immunologically induced renal disease.

I. Rasmussen, C. Nathorst Wingahl & J. Broberg (GÖRTELIN) INDUCED NEPHROPATHY IN RABBIT

C. Gustafsson, A. Chakravarty & J. Loh (ULTRASTRUCTURAL CHANGES IN MAST CELLS INDUCED BY ANTIGEN-ANTIBODY REACTION AND COMPOUND 48/80)

When a foreign protein together with pertussis bacilli is injected into rats, sensitizing antibodies develop in the blood within 10-12 days and cause sensitization of mast cells. If such cells from the peritoneal cavity are exposed to the sensitizing protein, histamine is released and many cells disrupt. In order to follow the ultrastructural changes during this process sensitized mast cells were incubated with the hemolytic antigen and stained with ruthenium red, an electron dense compound specifically reacting with and precipitating acid mucopolysaccharides. Histamine was determined using the biological method.

The most characteristic changes observed in the mast cells 5 minutes after the antigen-antibody reaction were enlargement and diminished electron density of the central granules. Generally the peripheral granules lost continuity with the cytoplasm and became relatively smaller and more electron dense. Complete dissolution of the granule with leakage of ruthenium red positive material was rarely seen. When it occurred it was interpreted as a late and final step. The synthetic compound 48/80 generally used to imitate anaphylactic histamine release produced similar changes in mast cells as the antigen-antibody reaction. The observed changes were therefore considered to represent early and essential features in mast cell anaphylactic injury.

H. Helmen, N. J. Freeman & S. Ögrenius (CELLULOSE HYDROLASE ACTIVITY AND ULTRASTRUCTURAL STRUCTURE DURING ENFORCED MAMMARY FEEDING AND INVOLUTION)

Current theories concerning the pathogenesis of involution mastitis are an active field. It is suggested in the literature that (1) increased activity of these enzymes with subsequent cell damage and (2) digestion of macrophages by proteolytically damaged cells or cell constituents (3) focal epithelial degeneration in secretory units.

In this study mammary gland involution was induced by removing the litter from lactating rats. Mammary gland excised during lactation or 1, 5, 10 or 20 days after the induction of involution were studied by electron microscopy. For electron fixation in OsO_4 or perfusion fixation with glutaraldehyde the formalin fixation of a cytoplasmic fraction of the cells was used. Biochemically the homogenates of lactating and involuting mammary glands were studied.

A rapid decline in gland weight, cell number and cell size occurred after the 3rd day. Significant alterations of the ratio of non-essential to total protein of the cytoplasm were not recorded. A moderate increase in specific alkaline phosphatase activity was noted at 5

ultrastructure of liver cells shows a proliferation of smooth ER only later. Smooth and rough microsomal subfractions exhibit a parallel increase of G6Pase activity in the first 5 hours. A second rise at 10-15 hours however first appears in rough and later in smooth microsomes. Three hours after alloxan injection k_m has changed from 2.5 to 4.9 (mM G6P). It remains at that level and is not influenced either by actinomycin or puromycin inhibitors of protein synthesis.

Incubation of microsomes *in vitro* in the presence of 0.02% per cent alloxan elevates G6Pase activity and results in a k_m of 5.1. When the enzyme is solubilized alloxan has no effect on the V_{max} or the k_m .

These results strongly suggest that alloxan in the liver influences primarily the membranes of the endoplasmic reticulum and thereby affects the kinetic properties of the enzyme. These events precede *de novo* synthesis of G6Pase in the liver of alloxan diabetic rats.

II CLAUMANN PHOSPHOLIPIDS AND NEUTRAL LIPIDS ISOLATED FROM LIVER MICROSOMAL SUBFRACTIONS

Cation containing sucrose gradients are useful in subfractionations of liver microsomes. In this way it is possible to obtain three subfractions: 1) rough microsomes (R), 2) smooth microsomes I (Sm I) and 3) smooth microsomes II (Sm II). The lipids of these fractions were analysed. The total microsomal fraction contains about 6.8 mg/g liver phospholipids (PI P) which corresponds to 85 per cent of the total lipids and 1.2 mg/g neutral lipids (NL) making up the remaining part. The subfractions have different PLP/protein ratios but this difference disappears after a washing procedure which removes the adsorbed protein and the luminal content. When the PI P's are separated on silicic acid impregnated paper the following distribution is obtained: phosphatidyl (pH)-ethanolamine 18 per cent, pH-serine 9 per cent, pH-choline 48 per cent, pH-inositol 11 per cent and sphingomyelin 5 per cent. The individual PI P's display different rates of incorporation. However the various subfractions exhibit the same patterns as well as specific activities upon comparison. The incorporation rate of glycerol C^{14} into total PI P is very similar in R and Sm I but significantly slower in Sm II. Separation of NL of total microsomes gives the following composition: cholesterol (Ch) 0.6 mg/g, cholesterol esters 0.07 mg, tri-glyceride (TG) 0.5 mg, free fatty acids 0.06 mg (Ch is somewhat concentrated in Sm I but particularly in Sm II). TG is twice as much in the smooth fractions as compared to the R. Measurement of total PI P after phenemal induced membrane synthesis gives high values for R and Sm I. On the other hand Sm II is constant and the absence of increase suggests a lack of participation in the process. It is concluded that the membranes of the Sm I are in many aspects similar but not completely identical with those of the R. Such a comparison results in an obvious difference in composition and turnover of membrane components of Sm II.

III HASSLER VASCULAR CHANGES IN SENILE BRAINS DEMONSTRATED WITH MICROARTHIOROGRAPHY

Three kinds of cerebral vascular deformities: 1) glomerular deformations, 2) vascular bundles and 3) wickercells have been studied in 231 autopsied cases. The changes were seldom observed in subjects below age 60 but were very common above age 70. They were almost equally distributed in the two sexes. They were not particularly correlated with high heart weight, high degree of cerebral arteriosclerosis.

or low brain weight. Vascular wickerworks were much more common in subject with vascular bundles than in those without. The glomerular loops occurred as a rule together with marked spiralling and might be products of excessive spiralling.

The influence of the vascular deformities upon the cerebral circulation was calculated partly on the basis of model experiments and partly by using *Poiseuille's* law. Each glomerular loop may reduce the blood flow in the particular artery by about 60 per cent. The vascular bundles and wickerworks probably reduce the flow to about 10 per cent of the capacity of a single vessel of the same diameter. Because the deformities only affect about one tenth of the arteries they probably do not influence the total cerebral blood flow more than the generalized spiralling. This affects almost all arteries in cases with glomerular loops and probably reduces the total blood flow by about 10 per cent.

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A Rinjerf ON CLASSIFICATION OF MAMMARY TUMOURS
SYMPOSIUM ON CELL PROLIFERATION AND ITS CONTROL IN VITRO

A Norrbj EXPONENTIAL AND CELL PROLIFERATION AS A FUNCTION
OF RATES OF BIRTH, DEATH AND GROWTH

Mathematical estimators of proliferation of cell populations increasing in size have been based on comparatively simple models of proliferation. Three factors may bias such estimator: a) cell death, b) a growth fraction less than one, and in some systems c) a feeder compartment where a proliferative class is not self-maintained but fed from some stem cell class. In tumour cell kinetics the significance of such a feeder factor is obscure. Growth fraction may be determined by cinemicrographic analysis of a monolayer culture. Cell abortion may occur during exponential growth in a population but has not been taken into consideration in mathematical estimators.

The aim of this study was to describe the growth of an established cell line *in vitro* whilst taking into consideration the above-mentioned bias, particularly cell death. We have assumed that an exponential net cell increase in an asynchronous population constitutes the difference between a more rapid exponential cell birth and an exponential and simultaneous cell death. Accepting this assumption it is possible to derive reasonably workable mathematical equations, and we have thus mathematically defined rates of growth, birth and death. These rates have been correlated to generation rates (reciprocal of generation times) recorded cinemicrographically in one and the same population and in three sister cultures.

The rates of birth, death and growth here presented give a satisfactory and a true description of the actual distribution of individual parameters in the cell populations.

G Moberg ON CLASSIFICATION OF TUMOURS OF THE URINARY BLADDER

Th Berge CARCINOMA METASTASIS AND EXTRAMEDULLARY HAEMATOPOIESIS IN THE SPLEEN

In 181 cases with carcinoma metastases in the spleen a correlation was found between extramedullary haematopoiesis and the degree of carcinomatous bone marrow replacement. But factors other than bone metastases are of importance and an increased frequency of extramedullary haematopoiesis was found only in cases with pronounced replacement of bone marrow (cancer involving all vertebrae). In cases in which some of the vertebrae only are involved extramedullary haematopoiesis was found to proceed at the same rate as in cases without skeletal engagement. The theories that tumour growth in the spleen might stimulate extramedullary haematopoiesis or vice versa were not supported by the investigation.

I Angervall, B Stener & I Wirlom CLEAR CELL SARCOMA OF TENDONS AND APONEUROSIS

In two cases of clear cell sarcoma of tendon and aponeuroses were described one of these apparently originating from the tendon of vastus medialis (23 year old man) the other from the tendon of tibialis anterior (60 year old man). This type of sarcoma was described by *Feninger* (1965) as a new tumour entity. The tumour is characterized by pale and uniform cells of epithelioid appearance arranged in compact nests and fascicles. The nuclei are pale staining but have a prominent nucleolus. Mitotic figures are scarce. The cellular aggregates are enclosed by delicate cuffs of fibrous connective tissue. The cysts are continuous with dense collagenous structures that transverse the tumour and merge with reformulated tendons and aponeuroses.

The most difficult differential diagnosis is synovial sarcoma. An important point of distinction is the total lack of high grade differentiation in clear cell sarcoma. This tumour also differs from synovial sarcoma as to the content of mucopolysaccharide and its clinical course is more protracted. Thus even though the ultimate prognosis is grave the 5 year survival rate after operative treatment is high.

K Værby, I Enerbæk & U Schelin DIFFERENTIAL DIAGNOSIS OF OVARIAN NEOPLASMS SIMULATING MALIGNANT BRENNER TUMOURS

Two cases of malignant ovarian neoplasms were reported both interpreted as primary ovarian tumours at operation later nullified due to tumour invasion in the uterus and the small intestine developed in both patients. They had 10 and 17 months respectively after the first operation exhibiting distant metastases.

Both ovarian tumours were solid and were of a similar morphological appearance with considerable cellular atypia. They were interpreted as possibly malignant Brenner tumours. The development of intestinal tumours led to a reconsideration of this diagnosis utilizing histochemical methods.

The tumour cells in formaldehyde fixed specimens from one of the cases showed histochemical properties strongly indicating a content of hydroxytryptamine (serotonin). Thus the tumour cells displayed reducing properties alkaline diazo coupling colour reaction with ninhydrin and fluorescence in ultraviolet light. In addition the presence of hydroxytryptamine was demonstrated by means of the *tryptophan* technique (*Enerbæk* to be published). All the histochemical reactions were negative in the other case which thus might represent a malignant Brenner tumour.

The findings clearly demonstrate the value of hist chemical reactions in the diagnosis of ovarian tumours simulating Brenner tumours and call attention to carcinoma as a diagnostic alternative in these cases

Chr Ahren, L Angerall, B Stener & I Stener OSSIFYING PSEUDO TUMOURS IN SOFT TISSUE

I Saurander & J Olsson MORPHOLOGICAL AND LIPID HISTOCHEMICAL STUDIES ON PERIPHERAL NERVES IN GLOID CELL LEUCODYSTROPHY (M. I. RABBE)

It is generally believed that peripheral nerves remain unchanged in globoid cell leucodystrophy (GLD). This opinion seems to be based on reported findings in only four cases of GLD in which peripheral nerves were said to be normal. But since the methods used and the examined regions of the peripheral nervous system were not stated the involvement of peripheral nerves in GLD must be considered an unsolved question.

We have examined various levels of the peripheral nervous system in six clinically and histologically typical cases of GLD. All the peripheral nerves (sciatic, femoral, sural and cutaneous nerves of the calf) presented severe degeneration of axons and myelin sheaths and neural fibrosis and perivascular accumulations of histiocytes. Spinal nerve roots and dorsal root ganglions were also severely damaged. Lipid histochemical studies of methylene blue in products in peripheral nerves from two cases revealed the accumulation of a PAS positive lipid most probably cerebroside in Schwann cells and endoneurial phagocytes. A presence of sudanophilic material in the nerves was not observed.

In Wallerian degeneration the myelin lipids including cerebroside are removed and sudanophilic cholesterol esters are formed. Thus the lipid histochemical pattern in our two cases of GLD is not of the Wallerian type but is most probably directly related to the underlying metabolic defect in GLD.

I Enerba ACTION OF SODIUM BOROHYDRIDE ON THE CHROMOGENIC MATERIAL IN CARCINOID TUMOURS

Sodium borohydride as a reducing agent in the histochemistry of metanamine was introduced by *Terri Hilla* & *Jonsson* who used it as a means by which the specificity of the formaldehyde gas method might be increased. Preliminary data on the effect of sodium borohydride and formaldehyde on the chromogenic material in carcinooid tumours fixed in formalin solution are reported.

The yellow fluorescence occurring in the tumour cells is not affected by borohydride treatment and partly regressed by formaldehyde treatment.

A modified ninhydrin procedure resulted in an orange colour in the tumour cells in a double reaction. Enterchromaffin inhibited a red fluorescence in the tumour cells. In the formaldehyde treated specimens the red fluorescence induced by formaldehyde treatment was not affected.

The findings suggest that the chromogenic material in the tumour cells is not a staining structure and that the formaldehyde treatment is not a necessary step in the formaldehyde treatment.

On the basis of these findings a new technique for the demonstration of 5-hydroxytryptamine in formalin fixed tissues is proposed. This technique utilizes the effect of borohydride and subsequent formaldehyde gas treatment on the yellow autofluorescence and the orange red fluorescence induced by ninhydrin and seems to offer a higher degree of specificity than that obtainable by methods hitherto available.

B. Boeryd, B. Hagmar & C. Johansson: EFFECT OF HEPARIN, FISH OIL AMINO CAPROIC ACID AND GUANETHIDINE ON INTRAVENOUSLY INDUCED METASTASES OF MELANOMA B16

In previous studies on the distribution and growth of intravenously induced metastases FACA increased the average and total metastasis volumes in the first capillary bed the lungs in two of three isologous systems. Apart from its antifibrinolytic effect FACA affects catecholamine metabolism. It shares this effect with guanethidine in which known antifibrinolytic activity is lacking.

It might be possible to evaluate the relative importance of the antifibrinolytic and sympathetic blocking properties of FACA by comparing the effects of FACA and guanethidine on metastasis formation.

Experiments were therefore performed on inbred C57Bl/6J mice with an isologous tumour Melanoma B16. An enzymatic tumour cell suspension was inoculated intravenously into controls, heparin, FACA and guanethidine treated mice.

In FACA treated as well as in guanethidine treated mice the number and total volume of metastases to the lungs increased while the average metastasis volume remained unchanged. Heparin decreased the average and total metastasis volumes to the lungs and the number tended to be reduced.

FACA and guanethidine had similar promoting effects on pulmonary metastases. Both drugs deplete the catecholamine stores. Presumably this effect somehow contributed to the increased number of pulmonary metastases after administration of FACA.

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SUBEPITHELIAL CHANGES IN ORAL SUBMUCOUS FIBROSIS

By

SATYAJATI M SIRSAT and J J PINDBORG

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Submucous fibrosis was reported as a distinct morbid entity of the human oral mucosa by Joshi (1953). He described the symptoms as vesicles on the palate in early cases gradually increasing oral fibrosis leading to trismus and a marked inability to eat food spiced with chillies. The identical oral state has been called variously as Atrophia idiopathica tropica mucosae oris (Schwarz 1952) "Diffuse oral submucous fibrosis" (Lal 1953) "Idiopathic scleroderma of the mouth" (Su 1954) "Idiopathic palatal fibrosis" (Rao 1962) and "Sclerosing stomatitis" (Behl 1962)—the interesting point being that except for Su's cases which occurred in the Chinese in Taiwan this condition has been reported only in Indians. Clinical aspects of the disease its histopathology and probable etiology have all been commented upon during the last few years (Sirsat & Khanolkar 1957 1960 1960a 1960b 1962 1962a DeSa 1957 George 1958 Sharan 1959 Pindborg *et al* 1964). A recent paper reports the epithelial changes that occur in the oral mucosa in submucous fibrosis (Pindborg *et al* 1965). This paper describes in detail the subepithelial changes that supervene in submucous fibrosis and assays a histological comparison in early and advanced stages of the disease.

MATERIAL AND METHODS

This study is based on biopsies obtained from two series of patients. One group of 8 cases was obtained from private or hospital ear nose and throat clinics in Bombay or from the admission clinics of the Tata Memorial Hospital, Bombay. The second group of 114 cases was collected from the Govt. Dental College, Lucknow, Bombay and Trivandrum. Most of the tissues were placed in 10% formalin. Fibrotic areas on the buccal mucosa. Occasionally an additional biopsy was taken from the palate gingiva or lip. Fixation was in 10% formalin, a buffer formalin.

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lin 6 μ thick serial sections were stained routinely with haematoxylin as in Mallory's trichrome (Weinmann & Meyer 1959) (Cridley 1957) Mallory's phosphotungstic acid haematoxylin Weigert's resorcin fuchsin (Harts modification) and toluidine blue at pH 4.5

RESULTS

On the basis of the histopathology in H & E sections the biopsies from submucous fibrosis can be grouped into four clearly definable stages 1) very early 2) early 3) moderately advanced and 4) advanced. These stages have not been judged only on the amounts and nature of the subepithelial collagen but on the following criteria in combination 1) presence or absence of oedema 2) physical state of the mucosal collagen 3) overall fibroblastic response (number of cells and age of individual cells) 4) dynamic state of blood vessels and 5) predominant cell type in the inflammatory exudate.

The four stages have been described as follows

1) *Very early*

Collagen finely fibrillar dispersed by the presence of marked oedema. Fibroblastic response strong with plump young cells with abundant cytoplasm. Blood vessels sometimes normal often dilated and congested. Inflammatory cells mainly polymorphonuclear leucocytes with an occasional eosinophil (Fig. 1).

2) *Early*

Juxtaepithelial area shows early hyalinisation. Collagen still seen as separate bundles which are thickened (this term is used to denote early coalescence within the bundle—which is still recognisable as a separate wavy unit). Plump young fibroblasts are present in moderate numbers. The blood vessels are often dilated and congested. The inflammatory cells are mostly mononuclear lymphocytes, eosinophils and an occasional plasma cell (Fig. 2).

3) *Moderately advanced*

Collagen is moderately hyalinised the amorphous change starting from the juxtaepithelial basement membrane. Occasionally thickened collagen bundles are still seen separated by slight residual oedema. The fibroblastic response is much less—the cells present being mostly adult fibrocytes with elongated spindle shaped nuclei and scanty cytoplasm. Blood vessels are either normal or constricted due to increased surrounding fibrous tissue. The inflammatory exudate consists mainly of lymphocytes and plasma cells though an occasional eosinophil is seen (Fig. 3).



Fig 1 Very early stage of oral cancer. The submucosa is thickened and dilated. Inflammatory cells are present. Haematoxylin and eosin $\times 155$.

Fig 2 Early stage of oral cancer. The submucosa is thickened and dilated. Inflammatory cells are present. Haematoxylin and eosin $\times 370$.



Fig 3 Moderately advanced oral submucous fibrosis. Moderate hyalinisation of mucosal collagen bundles. Thickened fibrils are visible and thin. Blood vessels narrowed. Haematoxylin-eosin $\times 210$.

Fig 4 Markedly advanced stage of oral submucous fibrosis. Mucosal collagen completely hyalinised, mostly acellular. Few vestigial fibrils in the profiles are seen. Blood vessels obliterated. Haematoxylin-eosin $\times 270$.

4) *Advanced*

Collagen is completely hyalinised and is seen as a smooth sheet with no separate bundles discernible. Oedema is absent. The hyalinised areas are devoid of fibroblasts though a thin elongated cell or vestigial nucleus is seen at rare intervals along the fibre bundle. Blood vessels are completely obliterated or narrowed. The inflammatory cells are lymphocytes and plasma cells (Fig. 4).

Table 1 shows the number of biopsies in the different stages of subepithelial response.

TABLE 1

Summary of Subepithelial Response in 199 Cases of Oral Submucous Fibrosis

Investigators	Place	Total No of cases	Stage of subepithelial response			
			Very early	Early	Moder- ately ad- vanced	Ad- vanced
Pindborg et al	Lucknow	32	1	20	8	3
Pindborg et al	Bombay	42	5	17	15	4
Pindborg et al	Trivandrum	40	1	17	17	5
Sirsat & Khanolkar	Bombay	85	—	30	36	19

The following Indian doctors co-operated in collecting the material: T. V. Chawla, D. Gupta, H. K. Kalapessi, S. A. Kale, B. Singh, A. V. Srivastava, B. V. Talparchan and J. Zachariah.

The fibrous connective tissue has been studied by three differential stains—Mallory's trichrome, Mallory's phosphotungstic acid haematoxylin (PTAH) and Weigert's resorcin fuchsin. The tinctorial changes of the collagen in submucous fibrosis as seen in serial sections have been evaluated on the basis of combined reaction to all the three stains. Described in sequence of severity of alteration, the different patterns of response are as follows:

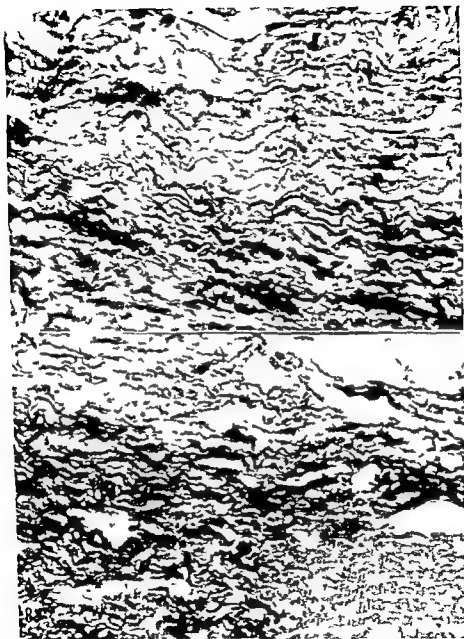
- 1) Dense thickened collagen staining evenly blue and evenly orange with the two Mallory methods and negatively with resorcin fuchsin.
- 2) Uneven pale to intense staining with the two Mallory techniques, negative to resorcin fuchsin.
- 3) Chromophobic or pale staining areas with the two Mallory stains (Fig. 5) with identical areas in serial section filled with resorcin positive thickened coarse fibres but granules (Fig. 6).
- 4) Identical fibre bundles partially or fully yellow or yellow-red with the trichrome, partially or fully purple with PTAH (Fig. 7) and with mild, moderate or massive amounts of resorcin positive material of the altered fibre bundles (Fig. 8).
- 5) Coalesced hyaline masses staining yellow with the trichrome, purple with the PTAH and positive with resorcin (Figs. 9, 10, 11, 12).



Figs 5 and 6

Subepithelial = flagon showing chr in phloem with I-TAH (Fig 5) and marked in
 cres = in resorcin positive fibrous material in identical area in serial section (Fig 6)

Fig 5—Mallory's PTAH $\times 360$ Fig 6—Weigert's resorcin fu hsin $\times 360$



Figs 7 and 8

Subepithelial collagen (Fig 7) — wing uneven pale to intense red positive serial section trichrome $\times 360$ F₆ — Weigert's crystal violet fibres in a fuhsin $\times 360$

This basic tinctorial alteration has been qualitatively classified as mild moderate or massive alteration according to how much total area in the section shows tinctorial change Table 2 shows the number of biopsies in each group in relation to the histologic grading seen in H & E sections

TABLE 2

Reaction of Subepithelial Collagen to Differential Stains for Connective Tissue in Submucous Fibrosis of the Oral Mucosa

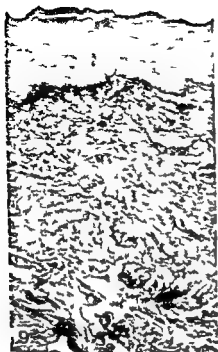
Histological stage	Tinctorial reaction of collagen to differential stains for connective tissue				
	No alteration in staining	Uneven staining no altered	Altered staining in occasional bundle	Altered staining in many bundles	Massive altered staining
Very early	—	—	1	3	—
Early	3	12	17	23	4
Moderately advanced	—	6	11	18	11
Advanced	—	—	2	8	13

DISCUSSION

Table 1 lists the biopsies in the four histological stages—very early early moderately advanced and advanced In the first group of 114 cases collected by Pindborg and his co workers 7 show very early and 54 show early changes Fifty two are in the moderately advanced or advanced group In the cases studied a few years earlier by Svirid & Akhanolkar (1962) there are no biopsies showing a very early response 30 show early changes while as many as 55 of the 85 are moderately or markedly advanced This marked difference in the grade of subepithelial response in the two series is probably due to two reasons The criteria used to recognise the disease in the 85 cases in the first group were marked fibrosis or trismus A majority of biopsies in this group were obtained from ear nose and throat clinics where the patient came to seek relief from the acute distress caused by the clinical complications of submucous fibrosis The second group of 114 biopsies was collected from dental clinics and the presence of a blanched mucosa or palpable fibrous bands even in clinically asymptomatic patients was considered sufficient indication of the disease to take a biopsy It is not surprising therefore that with such different clinical

Figs 9 10 11 and 12

1—Isinorubicin hyaline masses (Fig 10) staining well with Mallory's (Fig 9) purple with Mallory's PTAA (Fig 12) and showing retraction in identical area in serial section (Fig 11) Fig 9—Haematoxylin 10—Mallory's trichrome $\times 125$ Fig 11—Mallory's PTAA $\times 125$ Fig 12—Wiegert's trichrome (Fig 9) the material in $\times 125$ Fig 10—Mallory's trichrome $\times 125$ Fig 11—Mallory's PTAA $\times 125$ Fig 12—Wiegert's trichrome $\times 125$



hase lines *Sirsat & Khanolkar* 85 cases and the cases collected by *Pindborg* and his colleagues show such a marked difference in the distribution of histological stages of response

In 34 cases more than one biopsy was taken from clinically affected areas on the same buccal mucosa. An analysis of the histological grading in the multiple biopsies shows that in 22 patients both or all three biopsies showed a similar severity of histological response. In 12 patients however the biopsies from different areas showed difference in stages of histological response early in one tissue and moderately or markedly advanced in the other. This can be explained on the basis of focal initiation of the disease process in the mouth and therefore a natural unevenness in severity of the subepithelial change in different parts of the mucosa.

Normal collagen stains an even blue and even orange with the two Mallory stains. It is negative to resorcin which usually demonstrates the other fibrous scleroprotein of connective tissue elastin. In this study 3 biopsies, while showing densely thickened collagen typical to this disease stained normally and 21 showed uneven staining, especially in the deeper parts of the mucosa but with no abrupt tinctorial change. While this uneven staining is probable the earliest sign of collagen alteration it has not been considered as much as it is found though very occasionally in tissue believed to be normal. On the basis of altered staining reactions in submucous fibrosis the initial temptation is to suggest frank fibrinoid alteration of the submucosal collagen. Recent work on the immunohistochemistry of fibrinoid denotes the presence of a number of diverse vascular and sensitivity mechanisms (*Gillin et al* 1957 *Laques & Dixon* 1937 1958). Knowledge of the primary patterns of tissue response in submucous fibrosis is still too meagre to take for granted any such physiological sequence of events. *Unna* (1896) has described staining changes similar to those seen in submucous fibrosis in aging or degenerating collagen and called this altered material collagen. More recently *Gillman* (1955) and his group have studied altered collagen in skin and vascular endothelium and also find abnormal staining similar to that seen in submucous fibrosis. They call this marked transformation pseudoelastic or "elastotic degeneration". Till the alterations seen in submucous fibrosis have been characterised more minutely at molecular levels with the sophisticated techniques available today this histochemically descriptive term serves the purpose well. It is also in keeping with the submicroscopic degradation of altered collagen into structures similar to the dual forms of elastin (*Sirsat* 1958 *Sirsat & Khanolkar* 1960) and its susceptibility to digestion with crude chymase (*Sirsat & Khanolkar* 1960). Age of the host in relation to the amounts of tinctorial alteration seen in the oral mucosa collagen is an important parameter to consider in evaluating changes specific to the disease. Table 7 shows the amounts of change seen plotted against the ages in decade of patients. There is

no selective correlation between the age of the patient and the amounts of tinctorial alteration in collagen with the differential stains for connective tissue

The subepithelial reaction seen in submucous fibrosis is a continuous chronic inflammation and residual oedema with simultaneous attempts at repair with fibrosis. This coexistence of a defense as well as reparatory mechanism is termed a chronic productive response (Payling Wright 1954). The conditions under which a non viable agent can excite this response are when its action may be mild or its concentration too low to produce an immediate severe injury. Such an agent then operates more or less continuously for a long period with only moderate severity and the resulting tissue reaction assumes a more complicated pattern than is found with either acute inflammation or with tissue repair alone (Payling Wright 1954). When the nutritional state of the mucous membrane is defective the local defences of the host are retarded and any mild injury instead of ending in rapid uneventful healing may result in chronic inflammation. In moderately or markedly advanced biopsies of submucous fibrosis the blood vessels show a narrowed or blocked lumen due to increasing pressure of dense fibrous tissue (Sirsat & Pindborg 1967a). This undoubtedly results in impaired nutrition to all the oral mucosal elements. This point as also the overall malnutrition of the host system in the poor Indian patient has to be borne in mind when assessing the persistent mild to moderate chronic inflammation found in a majority of biopsies.

TABLE 3

Summary of Correlation between Age of Patient and Altered Collagen in 120 Biopsies of Oral Submucous Fibrosis

Degree of change in collagen	Age (in years)						Total Biopsies
	21-30	31-40	41-50	51-60	61-70	71-80	
No change	2	1	—	—	—	—	4
Uneven staining	5	4	4	6	7	—	21
Altered staining in occasional bundle	3	9	7	10	3	—	32
Altered staining in many bundles	4	7	20	10	6	1	48
Massively altered staining	5	8	8	9	—	—	30

The distribution of cell types in the inflammatory exudate in early and advanced biopsies raises a few interesting points related to the functional relationship of the leucocyte to the reaction in the tissue. In very early and mild cases the main cells are lymphocytes and polymorphonuclear neutrophils. The emigration of the cell from the blood stream into tissue spaces is an established reaction to injury in non-immune acute inflammation in man. However with mild to moderate

icity of tissue response and with the probable operation of other in direct systemic factors there is an increase in tissue eosinophils which remain in residual numbers even in moderately advanced biopsies. In the moderately or markedly advanced cases the predominant inflammatory cells are lymphocytes and plasma cells. The lymphocyte is believed to have a multipotential role in a trophocytic storage of protein (Kelsall & Crabb 1958) in fibroplasia through transformation into fibroblasts (Dumont 1959) and in the probable production of tissue immunity (Gowans 1962). While these interrelationships still merit further experiment the parallel presence of large numbers of lymphocytes and fibroblasts as well as plasma cells in moderate numbers emphasise the importance of sustained lymphocytosis in the maintenance of the tissue reaction in submucous fibrosis. Early and persistent tissue eosinophilia as also the presence of plasma cells also make it worth while to investigate further the earlier suggestion that the pathogenesis of submucous fibrosis of the oral mucosa might lie in a mild immune response (Sirsat 1959, Pindborg & Singh 1965). Preliminary studies on this basic facet of the disease are in progress.

SUMMARY

One hundred and ninety nine biopsies of oral submucous fibrosis have been studied by haematoxylin eosin and differential stains for connective tissue. The disease can be classified into very early, early, moderately advanced and advanced stages on the basis of the histological response. The nature of connective tissue changes, the persistence of mild to moderate chronic inflammation and the distribution of cell types in inflammatory exudate make it worth while to investigate an earlier suggestion that the pathogenesis of oral submucous fibrosis might be in a mild immune response.

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MAST CELL RESPONSE IN EARLY AND ADVANCED ORAL SUBMUCOUS FIBROSIS

By

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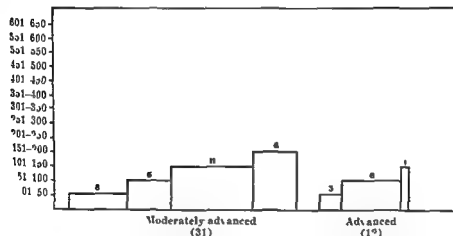
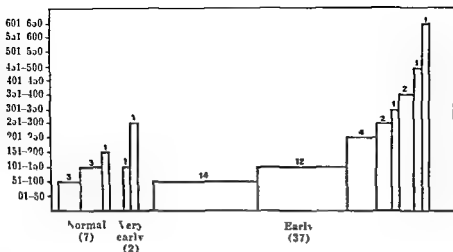
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The clinical and pathological features of oral submucous fibrosis have been described by a number of workers (Joshu 1953; Ial 1953; Su 1953; Sirsat & Khanolkar 1957, 1960, 1962; De Sa 1957; Rao 1962; Pindborg & Singh 1964; Pindborg & Zachariah 1965). Recent reports show the disease is fairly wide spread in all parts of India (Pindborg & Sirsat 1966). A significant correlation between the parallel presence of submucous fibrosis and cancers of various sites in the oral mucosa has also been noted (Paymaster 1957; Pindborg & Zachariah 1965). The aetiology of this condition remains obscure though the slow irritant action of capsaicin (the irritant principle of chillies—*Capsicum annuum*) has been assessed on the rat oral mucosa as a possible causative factor (Sirsat & Khanolkar 1960a, 1960b). The subepithelial response in oral submucous fibrosis (Sirsat & Pindborg 1967) as also in the vesicles which occasionally erupt is an early clinical sign (Pindborg & Singh 1965) suggest a rather complex pathogenesis. Biopsies from patients with this disease are therefore being studied from various aspects towards understanding the basic reactions in it. This paper reports the mast cell response in histologically early and advanced stages of the disease.

MATERIAL AND METHODS

Biopsies from 80 cases of submucous fibrosis were available for the study. The origin of the biopsies, the manner in which they were obtained and processed have all been described in detail (Sirsat & Pindborg 1967). From a thick paraffin section were stained with haematoxylin and eosin in order to establish the diagnostic and histological stage of the disease. Serial sections were stained with 1 per cent toluidine blue at pH 4.5. The mast cell counts were carried out on each section on one hundred 10×45 microscopic fields. Great care was always taken to maintain uniformity in counting starting from the right upper edge of the section in every case.

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Mast cell response in 80 biopsies of oral submucous fibrosis
(figures in brackets indicate total cases in each histological stage)

RESULTS

An earlier paper (Sirsat & Pindborg 1967) grades the subepithelial response in submucous fibrosis into four stages 1) Very early 2) Early 3) Moderately advanced and 4) Advanced. Fig. 1 shows the mast cell counts in 7 samples of normal buccal mucosae, 2 very early, 37 early, 31 moderately advanced and 12 advanced biopsies. The range seen in the seven normal oral tissues is from 51-100 to 151-200. Of the two very early cases, one shows a count of 101-150 and the other between 201-300. In the 37 early cases, twenty-six are within the range shown by the normal tissues, they have mast cell counts from 201-250 to 351-400. Two biopsies show a massive increase in mast cells, one in

451-500 group and the other in the 601-650 group. The moderately advanced biopsies all show mast cell counts either similar to normal or as in 8 biopsies even lower than in normal tissue. Similarly so in the advanced cases where out of 12 cases three are 1-50, 8 in the 51-100 group and one in the 101-150 group.

DISCUSSION

The ideal human buccal mucosa for assessing normality would be from persons in whom no trauma ever occurred. That however is not such a practicable event as apart from the common adult habits of smoking and chewing tobacco or the lime-betel mixture in the East the oral mucosa is exposed to sudden variations in temperature and repeated injury during mastication. The seven biopsies taken from buccal mucosae apparently free from disease show a range in mast cell counts from 51-100 to 151-200. This range has been considered normal for purposes of comparison.

The qualitative aspect of mast cells usually described is either the structural integrity with retention of the metachromatic granules or partial or complete degranulation of the cell. The significance of loss of granules from the mastocyte cytoplasm is still a matter of debate. Some workers (*Devitt et al* 1940) believe that mast cell degranulation is a sure morphological indicator of histamine release. Others feel that this phenomenon is a non-specific one brought about by many factors (*Selye* 1965) including hydration and mechanical injury (*Empeck* 1955). Degranulation or its absence has not been commented upon in this study. In counting partially degranulated cells obviously mastocytes were also included. An interesting observation was that in a majority of sections classified histologically as early with much vascular dilatation and oedema the mast cells were mainly perivascular. In the moderately advanced and advanced tissues the few cells found were scattered randomly in the tissue.

The pattern of mast cell response in oral submucous fibrosis is consistent with the overall sequential tissue response in the disease. In the early cases where reaction of the tissue to the irritant is the strongest the mast cell counts are highest. As the tissue gets converted to less reactive hyaline occasionally degenerating mass the mast cells become less and are often even fewer than in the normal mucosa. It is interesting to compare this response to that seen in inflammation. There is general agreement that in acute inflammation there is a decrease in tissue mast cells (*Leever* 1961). In the chronic state where there are cyclic incidents of necrosis or alternating proliferative and degenerative changes the mast cell counts are higher than those in normal tissue. According to *McCullocheon* (1953) in chronically increased vascularity and oedema decrease while mast cells increase in number. In oral submucous fibrosis as the disease advances there

is a drop in the number of mast cells being even fewer than found in the normal buccal mucosa. This is probably so because as the morbidity progresses the actively vascular oedematous connective tissue alters gradually to a smooth inert hyaline mass which does not encourage the maintenance of cell life. Active degranulation of the mast cells with continuous histamine release in the early stage might also account for the paucity of mast cells in the more advanced stages of the disease. Vascular dilatation and persistent oedema are predominant features of early stages of submucous fibrosis. These symptoms along with the high mast cell counts are tissue reactions similar to those described in certain mesenchymoses believed to be of autoimmune origin (Asboe Hansen 1964). If there is a trigger substance which elicits the marked increase in this histamine synthesising cell in sensitive human subjects the need to recognize it is very clear. The mast cell response in relation to the overall subepithelial response (Sirsat & Pindborg 1967), the presence of circulating and tissue eosinophilia (De Sa 1957, Rao 1962, Pindborg & Singh 1965) and the gammaglobulinaemia reported by some authors (De Sa 1957) all hint at the possibility that the pathogenesis of oral submucous fibrosis might lie in the realm of immunopathology. Further investigations are in progress in order to decide whether this is really so.

SUMMARY

Mast cell counts were carried out on toluidine blue stained sections in 80 biopsies of oral submucous fibrosis and 7 normal control buccal mucosae. One hundred 10×45 microscopic fields were counted in each section. In the early cases 9 out of 37 biopsies showed a significant rise in the mast cell population. In the very early moderately advanced cases they were similar to or less than those seen in the normal mucosa. The results are discussed from the viewpoint of the probable pathogenetic mechanism of the disease.

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THE VASCULAR RESPONSE IN EARLY AND ADVANCED ORAL SUBMUCOUS FIBROSIS

By

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Received 29 iv 66

Oral submucous fibrosis has been recognised as a distinct clinical and pathological entity (Sirsat & Khanolkar 1962 Pindborg & Sirsat 1966). The etiology and pathogenesis of this disease have not been fully understood as yet. Attempts to do so are being made by studying the basic tissue reactions in this oral disorder. This paper reports the vascular response in early and advanced stages of the disease.

MATERIAL AND METHODS

One hundred and twenty four biopsies were available for this study from patients in which a clinical and histological diagnosis of oral submucous fibrosis had already been made. Serial sections were stained with haematoxylin and eosin to establish the histological stage of the disease and Weigert's resorcin fuchsin for elastin (Hart's Modification) to observe the vascular elastic fibres.

RESULTS

The criteria used to grade the tissue response in oral submucous fibrosis have been described in detail (Sirsat & Pindborg 1967). In the biopsies studied 4 were in the very early stage, 61 in the early stage, 30 in a moderately advanced and 9 in an advanced stage. Among the bases used to grade the histological stage of the disease was the state of the blood vessels which appeared to show extreme dilatation in the early stages and narrowing in the more advanced stages. A detailed study shows however that the pattern of blood vessel reactions is more complicated than initially thought of and normal dilated and constricted vessels are found in the same section (Figs 1-4). The response has been therefore assessed in six groups according to the type of single or combined blood vessel reaction present. These groups are normal, normal dilated, dilated, constricted, normal constricted and constricted. The accompanying histogram (Fig. 5) shows the pattern of response in the 124 sections of submucous fibrosis observed. In the thirteen

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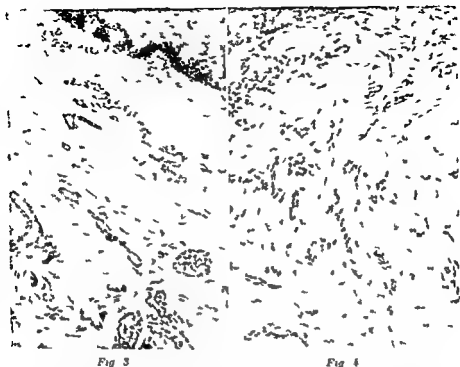


Fig 3

Fig 4

- Fig 3 Section from mucosa in oral submucous fibrosis showing hyalinised connective tissue. The blood vessels in the upper mucosa show constriction. Those in the deeper layer are dilated some exhibiting stasis. H & E. $\times 215$
- Fig 4 Section from mucosa in oral submucous fibrosis. There is marked constriction of the lumen in all blood vessels some showing complete obliteration of lumen. H & E. $\times 215$

This could be either due to the presence of mild subclinical inflammation or a greater sensitivity of these two subjects to local infiltration with anaesthetic prior to biopsy. In none of the 13 was there any constriction of the blood vessels seen—though one showed a mild hyalinisation of the connective tissue.

The microvascular aspects of tissue injury with increased permeability of the endothelium and dilatation of the vessels accompanying inflammation are only too well known. In oral submucous fibrosis inflammation is very commonly found coexistent to the connective tissue repair processes. It was necessary therefore to analyse the vascular response in terms of the inflammatory exudate present. Table 1 shows the types of vascular reactions seen in the 124 biopsies where inflammation is absent, minimal, mild, moderate, marked or massive. In 14 biopsies where inflammation has subsided completely, one shows persistent dilatation of all blood vessels, 11 shows dilatation of a few vessels and 1 constricted ones, can be a normal constricted reaction while in two all vessels are in various stages of constriction. Of the

COMPARISON OF VASCULAR RESPONSE IN RELATION TO HISTOLOGICAL STAGE OF ORAL SUBMUCOUS FIBROSIS

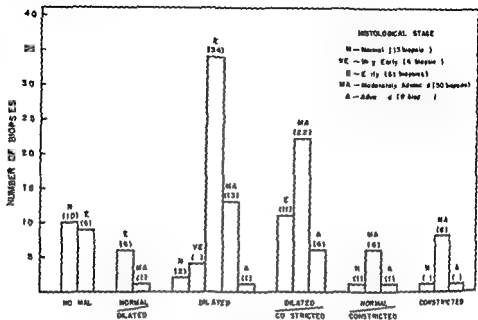


Fig 5

Histogram showing comparison of vascular response in relation to histological stage of oral submucous fibrosis

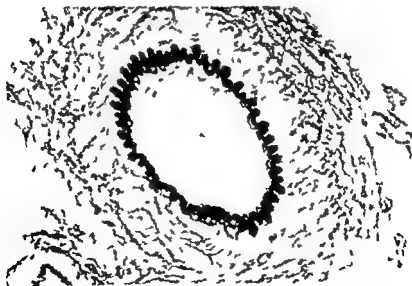


Fig 6

Section from mucosa in oral submucous fibrosis showing a deeper arteriole with a healthy muscular elastica (Weigert's resin fuchsin)

TABLE 1

Summary of Vascular Response in Relation to Inflammation in 195 Biopsies of Oral Submucous Fibrosis

Histo- logical stage	Inflam- matory exudate	Number of biopsies	Vascular response					
			Normal	Normal/ dilated	Dilated	Dilated/ constricted	Normal/ constricted	Constricted
Very early (4)	None	—	—	—	—	—	—	—
	Minimal	—	—	—	—	—	—	—
	Mild	—	—	—	—	—	—	—
	Moderate	4	—	—	4	—	—	—
	Marked	—	—	—	—	—	—	—
	Massive	—	—	—	—	—	—	—
Early (61)	None	3	1	1	—	—	—	1
	Minimal	3	2	—	—	1	—	—
	Mild	25	5	5	12	3	—	—
	Moderate	13	1	—	8	4	—	—
	Marked	10	—	—	8	2	—	—
	Massive	7	—	—	6	1	—	—
Moderately advanced (50)	None	9	—	—	1	8	1	1
	Minimal	4	—	—	—	3	—	1
	Mild	16	—	—	2	3	3	6
	Moderate	18	—	1	9	7	1	—
	Marked	3	—	—	1	1	1	—
	Massive	—	—	—	—	—	—	—
Advanced (9)	None	2	—	—	—	2	—	—
	Minimal	1	—	—	—	—	—	1
	Mild	4	—	—	—	3	1	—
	Moderate	—	—	—	—	—	—	—
	Marked	2	—	—	1	1	—	—
	Massive	—	—	—	—	—	—	—

40 biopsies which had mild inflammation 14 had a marked dilatation of all blood vessels while 21 had a few or all vessels with narrowed lumen. In the 57 biopsies showing moderate to massive inflammation 46 showed massive dilatation following the natural sequence of events in a tissue reaction to trauma. The remaining 18 biopsies had a number of vessels in various stages of narrowing which was apparent first in the smaller vessels in the upper mucosa and spread gradually to the larger deeper vessels. This follows the pattern of hyalineisation in this disease starting initially in the juxtaepithelial zone and spreading downwards with increasing intensity of the metaplastic process. The constriction of the mucosal blood vessels is therefore an obvious result of the increasing pressure exerted by the densely fibrous or frankly hyaline environment. The significance of the impairment of nutrition to the tissue due to the limited blood supply lies in its effect on the overlying mucosal epithelium. The lack of optimum metabolic precursors could be responsible for the aberrant restless epithelium seen occasionally

and the frankly atypical one seen more uncommonly so in biopsies of the disease. This atypical epithelium if insulted continuously with tobacco or other superimposed injury could lead to oral malignancy (Pindborg *et al* 1966).

The persistent dilatation seen in many moderately advanced and advanced biopsies also merits some comment. In oral submucous fibrosis while there is a rise in mast cells in the earlier stages of the tissue reaction the counts are similar to those seen in normal mucosa or even lesser in the more advanced stages (Sirsat & Pindborg 1967a 1967b). This would suggest that similar to the situation seen in many delayed reactions the vasodilator amines synthesised by the mast cells do not mediate in the presence of the vascular dilatation and residual oedema seen in the more advanced stages of oral submucous fibrosis. Other factors seem to operate in the sustenance of this long term partial vascular response. As to what these factors are can be only a matter of speculation at this stage of our knowledge of the etiology of the disease. Two among the many that come to mind in the light of the histopathology of the disease are the globulin permeability factor (Wilhelm 1965) and the presence of an antigen antibody complex which is known to sustain increased vascular permeability and dilatation and provoke a hyaline degeneration of connective tissue (Dixon 1963 1965).

SUMMARY

One hundred and twenty four biopsies from oral submucous fibrosis were studied in detail for the vascular response in relation to histological stage of the disease. Normal dilated and constricted blood vessels were seen often in combination in the same section. Epithelial changes consequent to impaired nutrition due to narrowed blood vessels have been discussed—as also the probable causes of the persistent vascular dilatation and oedema seen in the absence of inflammation in some advanced biopsies.

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STUDIES BY THE NEW TEST ON CARCINOGENIC PROPERTIES OF FATS

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Studies on the effect of heated and otherwise altered fats as carcinogens by the new test were carried out by one of the present authors (Arffmann 1964) who found this short term test sufficiently specific for a screening of chemical compounds given in oil. Among the substances tested certain oxidation products especially ethyl linoleate hydroperoxide gave positive results. The new test was still positive for the product obtained by reduction of the hydroperoxide group to an alcohol group.

The observations have been followed up in a more systematic screening of oxidation products of fats by the new test. Several parameters were taken into consideration. Oleic acid was compared with linoleic, linolenic and arachidonic acids which possess two, three and four double bonds respectively in the 1,4 position. Elaeostearic acid with three double bonds in the 1,3 position and the polyene squalene having four 1,5 double bonds were tested. Ethyl esters and triglycerides of the acids were compared. Excepting elaeostearic acid all compounds were tested as native compounds after oxidation to a suitable peroxide content and after reduction of the peroxide groups. The results of the studies are reported in the following.

MATERIALS AND METHODS

Substances. Triolein, trilinolenin, ethyl linoleate and ethyl linolenate were from Unilever Research Laboratories Vlaardingen II. Some samples of triolein and trilinolenin used as basic controls were from The Hormel Institute, Austin, Minnesota, U.S.A. (All these substances were supplied and tested at the Department of Food Chemistry, Aarhus, Denmark.) Most of the triolein, trilinolenin and ethyl linoleate were 90 per cent pure. Samples of free linolenic acid were obtained monthly from Grindsted, Aker, Norway.

Ethyl arachidonate was a gift from F. Hoffman, Lausanne, Switzerland.

Ethyl oleate was prepared by A. Jart, The Danish Institute of Food Chemistry, Copenhagen.

A sample of squalene (practical Eastman 90 per cent) was from M. H. L. cholanthrene (practical grade) was from Sigm. Chem. Co. U.S.A. Squalene was from Sigm. Chem. Co. U.S.A.

This work was supported by a grant from the Danish Anti-Cancer League.

TABLE 1
Properties of Substances Tested by the Nwt Test

Substance	p.v.	Optical density	Absorption maximum mμ
Ethyl oleate	30	0.3	217
Ethyl oleate hydroperoxide	650	3.5	217
reduced ethyl oleate hydroperoxide	2	3.3	217
Triolein			
Triolein hydroperoxide	1250	3.4	215
reduced triolein hydroperoxide		3.7	215
Ethyl linoleate			
Ethyl linoleate hydroperoxide	1200	13.9	233
reduced ethyl linoleate hydroperoxide		8	233
Trilinolein	100	0.9	233
Trilinolein hydroperoxide	1300	10.9	233
reduced trilinolein hydroperoxide		11.5	233
Trilinolenin hydroperoxide	1800	11.9	233
Ethyl linolenate		1.5	234
Ethyl linolenate hydroperoxide	1600	12.9	234
reduced ethyl linolenate hydroperoxide		12.3	234
Trilinolenin	100	3	235
Trilinolenin hydroperoxide	1100	10.9	235
reduced trilinolenin hydroperoxide	40	10.1	235
Ethyl arachidonate		1	235
Ethyl arachidonate hydroperoxide	800	2.2	235
reduced ethyl arachidonate hydroperoxide		1.8	235
Cholesterol	0	0.1	234
Cholesterol hydroperoxide	900	7.2	234
reduced cholesterol hydroperoxide	0	7.2	234
Squalene	4	6.9	217
Squalene hydroperoxide	87	28.0	212
reduced squalene hydroperoxide	70	27.4	212
Tung oil		147	271

The table gives the peroxide content in μ equiv./g. substance (p.v.) and the optical densities for 1 g. substance/1 l. cyclohexane at absorption maximum.

Most of the peroxides were prepared by simple aeration with atmospheric air. The peroxide content was controlled at suitable intervals and the aeration discontinued when a level about 1000 μ equiv./g. had been reached.

Ethyl oleate and triolein were aerated until a peroxide content of about 200 μ equiv./g. only. The products were then dissolved in petrol ether and chromatographed on acid alumina. The hydroperoxides were eluted with diethyl ether.

Cholesterol was oxidized by ultraviolet irradiation in a quartz flask of a glacial acetic acid suspension in an atmosphere of oxygen. After a suitable amount of oxygen had been absorbed water was added. The precipitate was washed with water and air dried.

The reduction of peroxides was carried out with stannous chloride in ethanol as recommended by *Irwin & Vickell (1956)*.

Peroxide determinations were made by the colorimetric thiocyanate method with the exclusion of atmospheric oxygen (*Farind & Hartmann 1955*). The results are expressed as microequivalents of peroxide per g. fat (peroxide value, p.v.).

Ultraviolet absorption spectra of the substances dissolved in cyclohexane were recorded by means of a Beckman Ratio Recording Spectrophotometer.

The peroxide content (p.v.) and spectral properties (optical densities at absorption maximum for a solution of 1 g./1 l. cyclohexane) are summarized in Table 1.

Most of the substances were diluted with arachidyl oil to a concentration suitable for the injection. The dilutions were easily prepared by simple mixing. Cholesterol

and its derivatives however are sparingly soluble in arachid oil and since it was desirable to give also these substances in a readily available form in clear solutions only weak concentrations could be prepared. One per cent (w/v) solutions were obtained by vigorous mechanical stirring. Solutions of methylcholanthrene were prepared in the same way with additional heating.

Experimental procedures The technique of the new test has earlier been described in detail (Arffmann & Christensen 1961, Arffmann 1963, Arffmann 1964b). Since maximal positive reactions were found after about two weeks it was found reasonable to simplify the test by amputating all tails on the 15th day. Animals dying before were included if three days had elapsed after the injection. Of the whole effective number of experimental animals only 8 died before the 7th day.

Details concerning the single experiments are given in Tables 2-6. An upper limit for the strength of the test solutions is set by the necrotic lesions produced by high concentrations of the substances (Arffmann 1964a). To facilitate comparison the peroxides were tested in dilutions of a calculated p.v. of 600. The response in relation to the dose was further studied by giving more dilute (p.v. 200) or when toxicity was low or negligible more concentrated (p.v. 1100-1800) products. The reduced and the native compounds were diluted to match the higher or the highest concentrations of the peroxides and in some instances applied undiluted.

Each experiment comprised native and altered compounds within the same group of ethyl oleate and derivatives and triolein and derivatives and included negative and positive controls. With minor exceptions each compound was injected into 6 animals. As far as possible males and females were equally represented. If the quantity of substance and the number of animals were sufficient the experiment was repeated twice. In some instances concentrations were altered during this sequence so with the ethyl oleate group because of an unexpectedly high toxicity and with the squalene group because of similar signs on the 4th day after the injection if the first animals. Low peroxide values of ethyl linolenate hydroperoxide and of trilinolenin hydroperoxide were not tested because of scarcity of substances and animals.

RESULTS

The results are summarized in Tables 2-6. Positive reactions cover + and (+) responses (Arffmann & Christensen 1961) while doubtful reactions (\pm) are not included. Effective number of animals results from the total number by eliminating animals died before the 3th day after the injection and animals found unsuited because of severe necrosis, cadaverosis or skin inflammation. One animal with minimal remnants of the injected oil was also excluded.

Local toxic effect of the injected solutions was generally mild or absent. Severe necrosis of the tail was found in only 10 animals and these had to be discarded. One had been injected with lung oil 10 per cent, 11 had received ethyl ester or squalene hydroperoxides and 7 had received the reduced compounds. None of the triglyceride derivatives induced such necrotic lesions. For the comparison of a strongly toxic lesion with that of a positive and a negative reaction see Figs 1-3.

All hydroperoxides of the fatty esters gave positive results by the new test. When more dilutions of the peroxides were tested an increasing effect of ethyl oleate, ethyl linoleate and ethyl arachidonate with increasing p.v. was observed. The dependence on the peroxide content is also indicated by the results with triglycerides and squalene. The responses to the reduced compounds did not however differ significantly from those to the corresponding hydroperoxides. The positive results after injection of ethyl linoleate hydroperoxide and the corre-



sponding reduced compound are quantitatively in keeping with those obtained in earlier experiments (Arffmann 1964)

One of the objects of the investigations was to compare the effect of altered ethyl esters with that of similarly altered triglycerides. Derivatives of triolein showed to be distinctly less active than those of ethyl oleate. triolein hydroperoxide p.v. 1250 was the only active concentration. A tendency to a lower activity of the triglycerides was also shown by linoleic and linolenic acids. A negative result was obtained by triolein hydroperoxide p.v. 600. On the other hand a positive response was shown by native trilinolenin which weakens the significance of the strong reaction to peroxidized and reduced trilinolenin.

TABLE 2
Experiments with Oleate Triolein Compounds

Substance	Peroxide content (p.v.)	Test solution		Number of experiments	Total number of animals	Results Positive reactions Effective number of animals
		Strength in oil arach	Calculated p.v.			
Oleum arachidis				4	12	0/12
Ethyl oleate	30	30.8		2	10	0/10
Ethyl oleate	30	9.3		1	8	1/6
Ethyl oleate hydroperoxide	650	15.4	100	2	10	0/10
Ethyl oleate hydroperoxide	650	30.8	900	3	16	7/16
Ethyl oleate hydroperoxide	650	92.3	600	1	8	Necrosis
Reduced ethyl oleate hydroperoxide	9	30.8		2	10	5/10
Reduced ethyl oleate hydroperoxide	2	9.3		1	6	2/2
Triolein		48.0		3	18	0/18
Triolein		100.0		1	6	0/6
Triolein hydroperoxide	1250	16.0	200	3	18	0/18
Triolein hydroperoxide	1250	48.0	600	8	18	1/18
Triolein hydroperoxide	1250	100.0	1250	1	6	2/6
Reduced triolein hydroperoxide	0	48.0	0	3	18	1/18
3-Methylcholanthrene		0.5		7	18	13/18

Figs 1-3

- Fig 1* Absence of epithelial reaction after the injection of ethyl oleate 92.3 per cent in arachid oil. The oil droplets are represented by empty holes (20X).
- Fig 2* Epithelial hyperplasia and infiltrative downgrowth after the injection of ethyl oleate hydroperoxide p.v. 600 (40X).
- Fig 3* Deep necrosis of tail after the injection of ethyl oleate hydroperoxide p.v. 600. The ulcerative defect is covered by infiltrative epithelium. The case was discarded. (40X).

TABLE 3
Experiments with Linoleate Trilinolein Compounds

Substance	Peroxide content (p v)	Test solution		Number of experiments	Total number of animals	Results to live reactions Effective number of animals
		Strength % in oil arach	Calculated p v			
Oleum arachidis				2	12	0/12
Ethyl linoleate		37.5		3	17	0/16
Ethyl linoleate hydroperoxide	1600	12.5	200	3	16	1/16
Ethyl linoleate hydroperoxide	1600	37.5	600	3	16	7/16
Reduced ethyl linoleate hydroperoxide	0	37.5	0	3	17	4/15
Trilinolein	100	46.2		3	19	1/16
Trilinolein	100	100.0		1	6	1/6
Trilinolein hydroperoxide	1300	15.4	200	1	12	1/11
Trilinolein hydroperoxide	1300	46.2	600	3	21	0/19
Trilinolein hydroperoxide	1800	100.0	1800	1	6	6/6
Reduced trilinolein hydroperoxide	0	46.2	0	3	18	5/18
3-Methylcholanthrene		0.5		3	18	11/17

TABLE 4
Experiments with Linolenate Trilinolenin Compounds

Substance	Peroxide content (p v)	Test solution		Number of experiments	Total number of animals	Results to live reactions Effective number of animals
		Strength % in oil arach	Calculated p v			
Oleum arachidis				2	10	1/10
Ethyl linolenate		37.5		3	14	1/14
Ethyl linolenate hydroperoxide	1600	37.5	600	3	14	11/13
Reduced ethyl linolenate hydroperoxide	0	37.5	0	2	12	10/11
Trilinolenin	100	54.5		2	8	2/7
Trilinolenin	100	100.0		1	6	2/5
Trilinolenin hydroperoxide	1100	54.5	600	3	14	10/14
Trilinolenin hydroperoxide	1100	100.0	1100	1	6	6/6
Reduced trilinolenin hydroperoxide	40	54.5		1	6	1/5
Reduced trilinolenin hydroperoxide	40	100.0		1	6	6/6
3-Methylcholanthrene		0.5		2	12	10/11

TABLE 5

Experiments with Ethyl Arachidonate Compounds and Tung Oil

Substance	Peroxide content (%)	Test solution		Number of experiments	Total number of animals	Results Positive reactions Effective number of animals
		Strength % in oil	Calculated p.p.			
Oleum arachidis				2	8	0/8
Ethyl arachidonate		75.0		2	■	1/8
Ethyl arachidonate hydroperoxide	800	25.0	200	■	12	2/8
Ethyl arachidonate hydroperoxide	800	75.0	600	2	12	5/10
Reduced ethyl arachidonate hydroperoxide	0	75.0	0	2	12	5/7
3-Methylcholanthrene		0.5		2	12	8/12
Tung oil		10.0		2	12	8/11
Tung oil		25.0		1	6	5/5

A comparison of the four ethyl ester hydroperoxides indicates that oleate and linolenate have the strongest effect on the animals while linoleate and arachidonate exhibited lower activity. Turning to the reduced compounds we find the ethyl linolenate more active than the other three compounds. In contrast the results with the triglyceride hydroperoxides and the corresponding reduced compounds indicate an increasing effect with increasing number of double bonds of the component acids.

Results with altered squalene are shown in Table 6 and have been mentioned above. Responses were strongly positive. Cholesterol hydroperoxide and the reduced compound had no effect on the newt skin probably on account of the weak concentrations applied.

Tung oil was tested in three experiments (Table 5). Because of local toxicity the primarily applied 25 per cent dilution was replaced in later experiments by the less toxic 10 per cent dilution in arachid oil. In both instances the response was strongly positive.

In general injections with arachid oil and with unalt red fairs had no effect and so served their purpose as negative controls. A single positive reaction does not influence a negative judgment unless it can be reproduced (Arffmann 1964b). On the basis of this principle native triolein may be estimated as doubtfully positive while only trilinolenin has shown a weakly to moderately positive activity. The preparations of native trilinolenin and trilinolenin showed however a rather high peroxide content which may well explain the positive results. Injections with methylcholanthrene in all instances had a strongly positive effect and so confirmed the reactivity of the animals.

TABLE 6
Experiments with Cholesterol Squalene Compounds

Substance	Peroxide content (p %)	Test solution		Number of experiments	Total number of animals	Results Positive reactions Effective number of animals
		Strength in oil arach	Calculated p %			
Oleum arachidis				3	18	0/18
Cholesterol	0	10		3	18	0/18
Cholesterol hydroperoxide	900	10	9	3	18	1/18
Reduced cholesterol hydroperoxide	0	10	0	3	18	1/18
Squalene	4	50.0		3	18	1/18
Squalene	4	58.6		1	6	0/5
Squalene hydroperoxide	875	50.0	437.5	3	12	6/10
Squalene hydroperoxide	875	58.6	600	1	0	6/6
Reduced squalene hydroperoxide	70	50.0		2	12	9/12
Reduced squalene hydroperoxide	10	58.6		1	6	2/5
3-Methylcholanthrene		0.5		3	18	13/17

DISCUSSION

An inspection of the figures of Tables 2-6 shows quite conclusively that evidence of carcinogenic properties is shown by three groups of substances 1 The hydroperoxides 2 The corresponding reduced compounds 3 Tung oil

The rather close identity of the responses produced by the hydroperoxides and by equal amounts of the corresponding reduced compounds shows that the hydroperoxide group is not a prerequisite for the carcinogen like effect The hydroperoxide group is supposed to be converted into an alcohol group through the reduction with stannous chloride A reduction to an alcohol group *in vivo* could be a plausible explanation for the similarity between the responses of the hydroperoxides and the reduced compounds If so the active substances would be fatty acids having a hydroxyl group in a position to a double bond

In this connection it should be mentioned that van Duuren *et al* (1963 1965) found certain epoxides and peroxides carcinogenic to the skin of mice They found activity of 1 hydroperoxy-cyclohex-3-ene and mention that carcinogenicity has earlier been reported for two further $\alpha\beta$ unsaturated hydroperoxides Kotin & Falk in 1963 reported preliminary data of tumorigenic response in mice after exposure to selected organic peroxides the strongest effect being elicited by m- and p-tert-butyl isopropyl benzene hydroperoxide

On the other hand a strong effect was also shown in our experiments by tung oil which does not possess hydroxyl groups. The characteristic acid of this oil is elaeostearic acid with three conjugated double bonds. The isomeric linolenic acid has three methylene interrupted double bonds and is inactive. Conjugation however takes place during autooxidation of the fatty acids with more double bonds. The optical density at about 233 $m\mu$ gives an expression of the amount of conjugation (Table 1). The conjugation remains after reduction and about the same maxima of absorption and optical densities were found in the hydroperoxides and the corresponding reduced compounds. All conjugated fatty esters exhibited carcinogen like activity.

An effect was however also shown by the hydroperoxides of oleic esters and squalene and the corresponding reduced compounds. Conjugated double bonds are not formed during autooxidation of oleic acid or squalene and it can be concluded from the spectral properties that they did not contain significant amounts of conjugated dienes as by products or impurities either. On the other hand the hydroperoxides and the reduced compounds of oleic acid have much higher optical densities at 212-214 $m\mu$ than the corresponding non oxidized compounds. Spectrophotometric studies on the oxidation of oleic acid and its esters have been carried out by Holman *et al* (1945). Their spectra also show a higher extinction at about 200 $m\mu$ for oxidized ethyl oleate than for the fresh compound but they do not discuss the reason for the increased absorption at this wavelength.

A second kind of isomerization besides conjugation which also takes place during the autooxidation process is formation of trans isomers from the native cis compounds. A cis trans isomerization takes place during autooxidation of all unsaturated fatty acids and also tung oil contains double bonds in trans configuration. Although an estimate of the extent of the trans configuration in the products was not made there can be no doubt that a high degree has been present in all the active products.

The most thoroughly studied class of chemical carcinogens are the polycyclic aromatic hydrocarbons. Although this group of substances is chemically very distant from the altered fatty acids an important similarity between the two groups of lipid soluble substances exists. Both possess π -electrons. The π -electrons are probably in both cases responsible for the carcinogenic properties but also other requirements must be fulfilled. A special electronic configuration seems to be required for carcinogenic activity of the hydrocarbons but a complete agreement with chemical prediction has so far not been obtained. The possible importance of a hydroxyl group in α position to a double bond of conjugation of the double bonds and of trans configuration has been discussed above. The final identification of the configuration or configurations responsible for the activity can only be made by further screening of more substances.

The presence of a hydroxyl group generally cancels the activity of the hydrocarbons (Falk *et al* 1962) but the negative effect may be due to a more rapid elimination from the organism of the hydroxylated compounds. On the contrary, all our active compounds with the exception of tung oil contain a hydroxyl or a hydroperoxide group. Metabolic relationships may, however, also play a role for the activity of altered fats. The tendency to a smaller activity of the triglycerides in comparison with the corresponding ethyl esters may be due to a more rapid attack by lipase as the first metabolic step but of course also to different physico-chemical properties.

Due to the sparing solubility in arachid oil the conversion products of cholesterol were tested in only weak concentrations and no activity was found. Conversion products of cholesterol have been rather extensively studied for carcinogenic effects (Fieser 1964). The point of departure for the studies has generally been a similarity with chemical carcinogens with respect to ring structure. Our results indicate that the clue for the nature of carcinogenicity of lipids must rather be sought in the configuration around the double bonds in general and those of the fatty acids in particular.

Although the indication of carcinogenic properties of altered fatty acids has been obtained only by the new test it would seem to be of considerable interest that such minor configurative changes or substitutions in essential or ubiquitous substances of organisms as the essential fatty acids may result in dangerous qualities. The literature contains a variety of reports on toxic properties and suspected carcinogenicity of altered fats but studies of the effects of specific alterations of the fatty acids are more scarce. We found no great scale toxicity of parenteral injection of a variety of lipoperoxides in rats and chicks (Glamind, Söndergaard & Dam 1961). Rather high amounts of lipoperoxides are present in rat skin surface lipids (Glamind & Christensen). The effects of isomerized fatty acids have been studied especially in nutritional experiments in relation to essential fatty acids (Jørgensen 1966). The identification and separation of the isomerized or otherwise altered products of unsaturated fatty acids is made possible by recent methods. The possibility of carcinogenic effect might give new impetus to a study of the biology of altered fats based on chemically well identified substances and studies on these compounds may on the other hand throw new light on the mechanisms of carcinogenesis.

SUMMARY

Carcinogenic properties of derivatives of fatty acids and certain other lipids were studied by the new test. The ethyl esters of oleic, linoleic, linolenic and arachidonic acids, the hydroperoxides of the esters and the compounds obtained by reduction of the hydroperoxides were tested. All the hydroperoxides and the reduced compounds were active.

Slightly smaller activities were shown by the hydroperoxides of triolein, trilinolein and trilinolenin and the corresponding reduced compounds. High activities were shown by squalene hydroperoxide and its reduced derivative and by native tung oil.

The results are commented and the possible importance of a hydroxyl group in α position to a double bond of conjugation of double bonds and of trans configuration for carcinogenic properties of lipids is discussed.

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LACTIC ACID DEHYDROGENASE ISOENZYMES IN HUMAN MELANOMAS

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By combination of histochemical and electrophoretic techniques the demonstration of isoenzymes with substrate specificity is possible. Measurement of isoenzyme activities may yield more information than simple measurement of total activities as the individual isoenzymes may be altered in opposite directions leaving the total activity nearly unchanged.

Under pathologic conditions the cells of damaged organs may release some of their intracellular enzymes into the bloodstream. An increased amount of lactic acid dehydrogenase (LDH) in serum of patients with advanced malignant tumours were found by *Hill & Levi* (1954). Changes in serum LDH isoenzymes are seen in myocardial infarction with cell necrosis as demonstrated by *Wieme* (1959), *Taursen* (1962) and *van der Helm* (1962). In malignant effusions changes have been described by *Richterich et al* (1962) and alterations in the isoenzyme pattern of brain tumour homogenates have been shown by *Cerhardt et al* (1963). Patterns of LDH isoenzymes in tumours of the stomach, colon, mamma, kidney, lung, and prostate in 20 patients have been demonstrated by *Nissen & Bohn* (1965).

A number of different isoenzymes in amelanotic and pigmented melanomas of hamsters and one human pigmented melanoma have been investigated by *Pandov & Dilov* (1966). The average per cent of the LDH isoenzyme pattern of 7 human melanomas have been presented by *Bohn & Rothenborg* (1966).

The present investigation is an attempt to differentiate and compare the LDH isoenzyme pattern of pigmented human melanomas to their histology and histochemistry in relation to normal human skin with regard to lactic acid dehydrogenase.

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MATERIAL AND METHODS

Twelve pigmented primary malignant melanomas as defined by Allen & Spitz (1953) & cases of lymphnode metastasis and 12 skin specimens from different persons were investigated as to their contents of LDH isoenzymes. All primary tumours and the skin specimens have been painted with alcoholic iodine before surgical removal. Immediately after extirpation the tissues were carefully prepared for homogenization by an electric glass homogenizer being cooled by ice water. Subcutaneous fat was removed from the normal skin by scraping lightly with a knife in order to obtain comparable specimens. After centrifugation (10 000 r.p.m.) the clear supernatant was used for agar gel electrophoresis as described by Wieme *et al.* (1963). Samples were placed in Disco Cell Agar buffered to pH 8.6 by diem 1 sodium buffer. Two by two the slides were immersed in a bath of light petroleum for cooling purposes and electrophoresis was performed at 140 volt for 30 minutes. The slides were incubated at 37° together with frozen sections from the same tissues and stained by nitroblue tetrazolium DPX techniques using rubber frames as described by Bohn & Rothenborg (1966). The histology of the specimens was further controlled in haematoxylin eosin stained frozen and paraffin sections. The staining of the electrophoresis was fixed in acetic acid ethanol at room temperature for 120 minutes and dried. Scanning was performed at a Densicord (Photovolt) at 530 nm. The coloured bands were numbered from the anode and their enzyme activity expressed as percentage of total activity. All samples were run as duplicates.

TABLE I

LDH Isoenzyme Pattern in 12 Human Malignant Melanomas. Enzyme Activity Is Expressed as per cent of the Total Activity

Bands	I	II	III	IV	V
Slide No	Anode		Cathode		
16097/66	7	22	29	24	18
489/66	7	21	28	26	18
684/66	8	22	26	24	20
1016/66	3	17	31	29	18
1939/66	3	8	28	33	28
3170/66	0	3	22	36	39
4875/66	2	17	25	29	27
4821/66	2	15	26	28	29
6071/66	8	9	31	29	22
8241/66	3	14	30	29	24
6679/66	24	24	24	21	7
7773/66	8	9	33	30	23
Average per cent	7.1	15.1	29.0	26.6	22.7

RESULTS

Table I presents the results of the LDH isoenzyme activity of 12 human pigmented melanomas. The enzyme activity is shown as per cent of total activity. In all cases but two 5 bands of LDH activity were present. The main activity was found among the cathodal bands in all tumours. In 7 cases the greatest amount of activity was found in band 3 while 3 tumours appeared to show the greatest amount of isoenzyme in band 4 and 2 in band 5.

The relative activity of LDH isoenzyme distribution in lymphnode metastasis from malignant melanomas is given in Table II. It is seen

that the main portion of the isoenzyme pattern is found among the cathodal bands. In all cases 2 anodal and 3 cathodal bands are present.

Table 3 demonstrates the LDH isoenzyme pattern in normal human skin. It is seen that also for this specimen the main activity is found among the cathodal bands. It appears that none of the specimens investigated contained more than one anodal band.

TABLE 2

LDH Isoenzyme Pattern in 6 Lymphnode Metastases from 6 Human Malignant Melanomas. Enzyme Activity Is Expressed as per cent of Total Activity

Bands	I	II	III	IV	V
Slide No	Anode			Cathode	
15 157/66	14	22	21	21	18
79/66	5	7	29	39	17
4 001/66	13	22	31	24	10
5 435/66	20	21	26	18	11
11 884/66	7	21	31	25	16
11 071/66	5	10	34	1	20
Average per cent	10.7	17.9	29.2	23.3	17.0

TABLE 3

LDH Isoenzyme Pattern in 12 Human Skin Specimens. Enzyme Activity Is Expressed as per cent of Total Activity

Bands	I	II	III	IV	V
Slide No	Anode			Cathode	
4 875/66	0	8	21	26	41
4 891/66	0	4	27	22	37
4 891/66	0	0	36	26	38
4 903/66	0	2	29	32	33
5 062/66	0	21	36	43	0
5 166/66	0	15	47	16	2
5 840/66	0	2	45	53	0
11 071/66	0	0	23	31	41
11 146/66	0	0	58	42	0
11 38/66	0	0	31	47	22
6 241/66	0	0	22	32	36
6 541/66	0	0	19	43	38
Average per cent	0.0	4.3	33.7	37.0	25.0

The histologic control showed that all tumours investigated were malignant melanomas. The skin specimens did not present pathologic changes. The lymphnode metastases were all from patients with a primary histologically verified malignant melanoma and the microscopic pictures were of typical structure. The highest LDH activity was found in tumour cells compared with other types of cells present in the sections stained by histochemical methods.

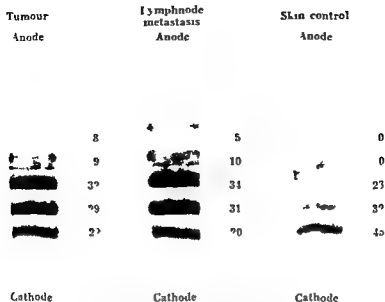


Fig. 1

The LDH pattern in primary tumour, lymphnode metastasis and skin control from the same patient (6071/66)

Enzyme activity is expressed as per cent of total activity on level with the bands demonstrated

DISCUSSION

Different types of LDH have been found by *Cahn et al* (1962) and by *Fine et al* (1963). The LDH isoenzyme pattern during fetal development has been described by *Philip & Vesell* (1962) in chick liver and muscle. The changes were characterized by a progressive increase of the cathodal bands relative to anodal bands. *Jensen & Thorling* (1967) suggested that changes in oxygen supply may alter the LDH pattern as they found that the isoenzymes in anaemic and cobalt treated rabbits were found to change gradually from prominence of the anodal to a prominence of the cathodal isoenzymes compared to control animals.

In malignant tissue *Gerhardt et al* (1963) described a more cathodic pattern of LDH isoenzymes from malignant brain tumour homogenates compared to normal brain tissue. Similar results are presented by *Nissen & Bohn* (1965) from investigation of animal biopsy material from tumours of the stomach, colon, rectum, liver, lung and prostate compared with controls.

In the present study the enzyme activity in the LDH pattern of malignant melanomas (Table 1) and normal skin (Table 2) showed a dominance of the cathodal isoenzymes. From Table 1 it appears that all melanomas except 2 showed two anodal bands. The average of these is essentially higher than the average of the skin controls where only 1 anodal band was demonstrated. All skin covered specimens were painted with alco-

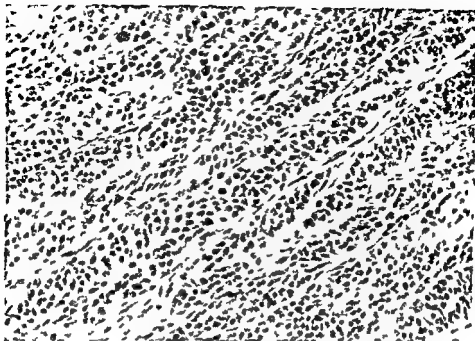


Fig 2

Fig 2 demonstrates the amount of fibrous tissue and inflammatory cells in melanotic tumor tissue used for homogenization (haematex slide in $\times 300$)

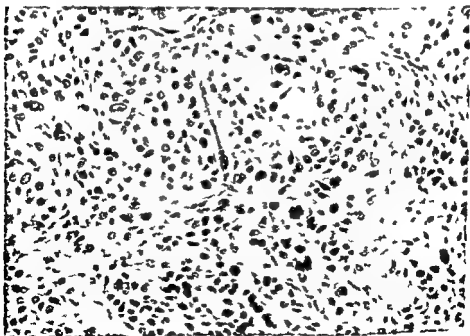


Fig 3

Fig 3 shows only small amounts of other cells in tumor tissue used for homogenization (Haematex slide in $\times 300$)

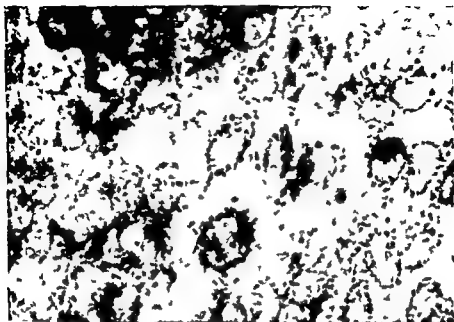


Fig. 4

A large amount of lactic acid dehydrogenase is demonstrated as colour precipitates in the cytoplasm of the tumour cells (Nitroblue tetrazolium $\times 3700$)

holic iodine but this comprised the surface of the tumours so that it does not explain the differences found. An increase of the anodal bands relative to the cathodal bands of the melanomas may therefore indicate an alteration of the LDH isoenzymes in proliferating tissues of the skin. This is supported by the investigation of *Bohn & Rothenborg* (1966) who in 8 basal cell carcinomas and 31 plantar warts also found a change towards the anode in the average LDH isoenzyme pattern compared with normal skin.

The LDH pattern in lymphnode metastases from malignant melanomas are given in Table 2. In all cases 5 bands of activity were present. Only 4 bands of activity, however, have been described by *Iandov & Dikov* (1966) in 2 cases of lymphnode metastases from melanomas. The LDH pattern of the lymphnode metastases investigated is very much alike that of the primary malignant melanoma. Fig. 1 demonstrates the LDH pattern in a primary tumour and lymphnode metastasis compared to skin control from the same patient. It appears that the total activity in skin judged from the summation of the bands is much less than in the tumour tissues. It is further noted that the values for enzyme activity in the melanoma and the metastasis demonstrated on Fig. 1 are very close. This may suggest a possible way of identifying metastasis from an unknown primary tumour among which the melanomas may give rise to very anaplastic and polymorphic metastases. As

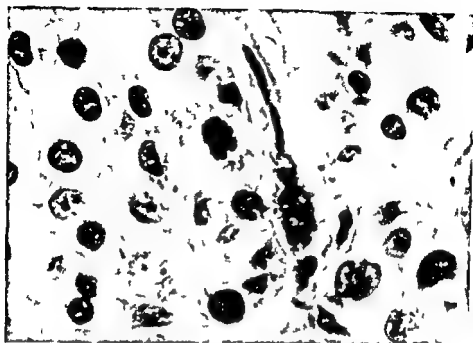


Fig. 5

The individual variation in size, shape and nuclear detail seen in some melanomas.
(Haematoxylin-eosin $\times 3,700$)

it appears from tables given the variation in figures is rather wide. Therefore the LDH isoenzyme pattern of a great number of tumours and metastases from the cathodol increasing group should be investigated and results not only presented as average values but with full attention to individual variations.

Finally, the histology of the tumours was compared with the patterns of LDH. No correlation was found with regard to the histological structures though the variation among the different types of melanomas investigated was large as demonstrated on Figs. 2 and 3. It is seen that the histologic control of the tumours revealed that to some extent also other tissues were present but to a varying degree. Thus fibrous tissue containing a number of inflammatory cells as necrotic neoplastic areas were found although grossly the tissue investigated appeared to consist of tumour tissue only. These findings however did not show any correlation to the isoenzyme pattern found which was dominated by the neoplastic lesion. This is in accordance with the histochemical studies of the LDH in sections from the same tissue. A large amount of enzyme was found in the tumour cells as demonstrated in Fig. 4. Although other cells than the cancer cells contained LDH, the precipitate of blue coloured formation was much more pronounced in the neoplastic cells which great variety in size and nuclear detail is demonstrated in Fig. 5.

SUMMARY

By means of agar gel electrophoresis combined with histochemistry using artificial electron transport systems as staining procedures (Nitro-BT DPN) the isoenzyme pattern of lactic acid dehydrogenase was examined in homogenates from 12 human pigmented melanomas 6 cases of lymphnode metastasis and 12 specimens of normal human skin as controls. The isoenzyme activity was expressed as per cent of total activity and the electrophoretic bands numbered from the anode. All tissues investigated had been controlled by light microscopy.

The melanomas were found to have an increase of anodal bands relative to cathodal bands compared with normal skin which may indicate alterations of the LDH isoenzymes in proliferating tissues of the skin. This is in contrast to several tumours of other origin where a relative increase of the cathodal fractions are found. The lymphnode metastasis showed an isoenzyme distribution similar to that of malignant melanomas. This may suggest possibilities of identifying metastasis of an unknown primary tumour among which the melanoma may give rise to very anaplastic and polymorph metastatic lesions.

The differences in histology of the tumours and lymphnode metastasis were not found to show correlations to the LDH pattern of the specimens investigated. The histochemically stained frozen sections gave evidence that the greatest amount of LDH was present in tumour cells compared to other cells found in the same section.

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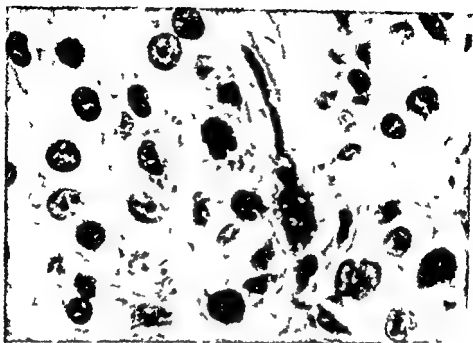


Fig. 3

The individual variation in size, shape and nuclear detail seen in some melanomas (Haematoxylin-eosin $\times 3900$)

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THE FINE STRUCTURE OF THE RENAL GLOMERULUS IN ACUTE ANURIA

By

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Received 9 ix 66

In acute renal failure the clearance values for the usual filtration substances are very low but give only minimum values for the size of the filtration because the possibility of a back diffusion of the ultra filtrate cannot be eliminated. Nevertheless it is the general assumption that glomerular filtration during the anuric phase is likely to be strongly diminished (Brun 1954).

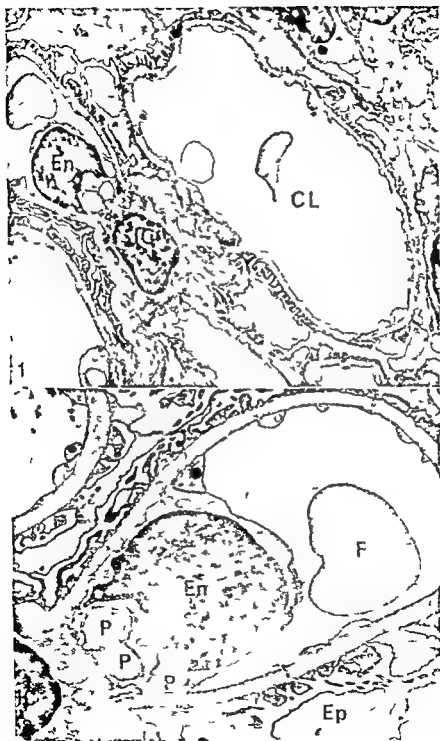
Light microscopic studies have not shown any pathological lesion of the glomeruli in acute anuria (Brun & Munck 1957). Electron microscopic investigations made in only a few patients showed glomerular components to be normal (Dalgaard 1960, Iaguen & Viatello 1964). Regnier (1950) in one case found endothelial hypercellularity. In order to extend and supplement these earlier investigations a study of 12 renal biopsies from 10 patients with acute renal failure was undertaken. The findings were compared with biopsies from 5 patients with normal kidney function.

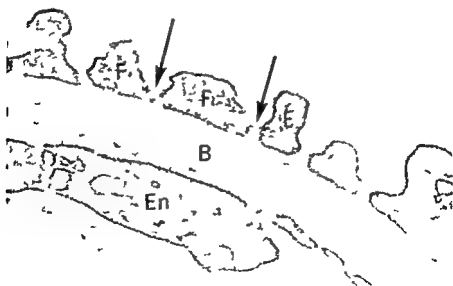
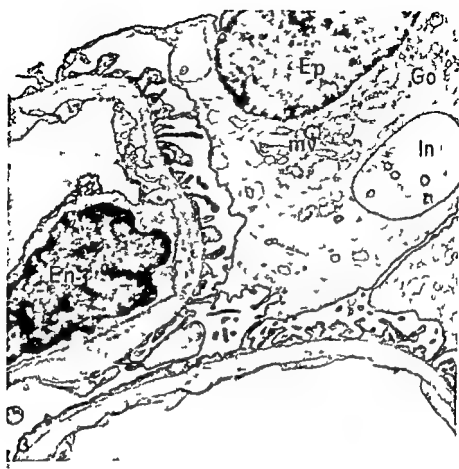
MATERIAL AND METHODS

The patients. Detailed descriptions of the clinical and laboratory data of the patients are published elsewhere (Steen Olsen 1967). The patients were between 20 and 69 years old. The acute renal failure appeared as a result of surgical or obstetrical operations in 1 patient, some of them complicated by sepsis or it followed poisoning with sulfonamides, barbiturate, or tranquillisers (Truxal) (3 patients). In 7 of the patients clinical manifestations of the disease were observed. The biopsies were performed between the 2nd and the 14th day of illness. Two of the patients were biopsied twice. Two biopsies were performed in the same patient. Eight patients had been treated with hemodialysis. All patients had received infusion of 0.9% NaCl glucose solution. Three of them had also been treated with Ringer's solution. Three of them had also been treated with Ringer's solution. Three of them had also been treated with Ringer's solution. The renal biopsies were performed under aseptic conditions through a small incision in the right lumbar region.

This work was supported by Danish Scientific Foundation and Michaelson Foundation.

We are indebted to civil engineer Mr. A. Birch and senior laboratory technician Mr. J. Pedersen for the photographic reproductions.





tained plasma and some red blood corpuscles. Thrombi or fibrin deposits were not present. The epithelial foot processes were not fused; the intervening filtration slits could be demonstrated. The basement membrane was of normal thickness and structure.

Some epithelial cells from cases of anuria as well as from controls contained 1–4 micron large inclusions in the cytoplasm consisting of vacuoles containing smaller homogenous rather electron dense globules and in some sections small amounts of granular material (Figs 5 and 6). The mesangial cells (intercapillary cells) were of normal size and structure. They were situated in a network of branches of basement membrane like material and often plump processes of rather clear cytoplasm could be seen projecting into the capillary lumina covered or uncovered by endothelial cytoplasm (Fig. 2).

The following deviations from the normal structure were noted.

In two biopsies (Nos 1 and 2 both from the second day of anuria) a swelling of the epithelial cytoplasm was observed which filled up the capsular space changing this to a narrow slit (Fig. 7). Normally some epithelial cytoplasmic processes are present in which the structure is uniform and of a medium electron density without organelles or ribosomes. These processes were the cell constituents which seemed to be expanded in the two cases. None of the other ten biopsies showed this phenomenon. It could not be demonstrated by light microscopy of Helly fixed tissue. One biopsy (No 3 3 days anuria) showed hyperplasia of the intercapillary cells and of the endothelial cytoplasm with narrowing of the capillary lumina (Fig. 8). The number of intercapillary cell nuclei seemed to be increased. No light microscopic changes were noted in this case; no mitoses of the intercapillary cells were seen. In all other biopsies the capillary lumina were patent and of normal dimensions. They sometimes contained red blood corpuscles in an amount which was equal to that of normal biopsy tissue.

In all biopsies from patients with anuria the abnormal presence of *cytosomes* in the epithelial cytoplasm and more seldom in the endothelial and intercapillary cells was noted (Figs 5, 6 and 8). They were of the same type as those normally present in many cells in the renal tubules of normal rats (Ericsson 1964). They could readily be distinguished from the normally occurring polyglobular vacuoles in

Figs 3–4

- Fig 3 Same case. Normal constituents of epithelial cytoplasm. Preserved foot processes, normal capillary. Ep. nucleus of epithelial cell. In normal occurring globular inclusion with dense bodies mv. multivesicular body. Go. Golgi zone. En. nucleus of endothelial cell. Glutaraldehyde fixation 15000 X.
- Fig 4 Biopsy No 9 eight days of anuria. Normal capillary wall with all constituents preserved. F. epithelial foot processes. B. capillary basement membrane. En. endothelial cell cytoplasm. Arrow points toward the basal part of filtration slit. Glutaraldehyde fixation 85000 X.



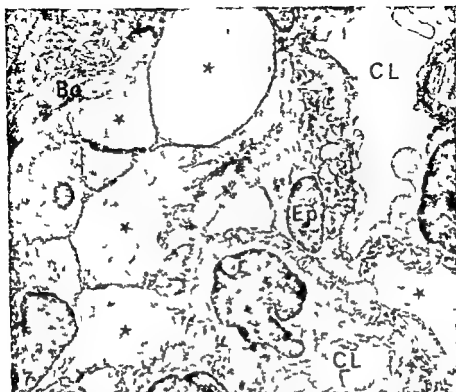


Fig 7

Biopsy No 2 second day of anuria Epithelial cytoplasm of uniform finely granular structure without organelles (marked by asterisks) occurs normally in human glomeruli but in this case it is swelled filling out the capsular space Bo basement membrane of Bowman's capsule CL capillary lumina Ep nucleus of epithelial cell Glutaraldehyde fixation 8000 X

the cytoplasm of glomerular epithelial cells. The cytosomes presented as single membrane bounded vacuolar structures of about $\frac{1}{2}$ to 2 micron. The space inside the vacuoles could be electron optically empty or finely granular with a few electron dense inclusions of variable shape granular fibrillar homogenous and concentric myelin like. Identifiable cell organelles or remnants of such were not observed. In some cases the electron dense matter totally filled the vacuoles.

Figs 5-6

- Fig 5 Cytosome (cytosome) is a membrane bounded structure. In the right of the cytoplasm, it is a vacuolar inclusion. It is normally occurring in the human glomeruli. To the left a part of an epithelial nucleus is visible. Biopsy No 7 5th day of anuria. Glutaraldehyde fixation 2500 X.
- Fig 6 Same case. The cytosome contains myelin like concentric material. Glutaraldehyde fixation 4300 X.



Fig 3

Biopsy No 3 third day of anuria Capillary lumen occluded by swelling of endothelium and intercapillary cells This phenomenon was observed only in this case Compare with Fig 2 Io nucleus of endothelial cell Io nucleus of intercapillary cell Cy cytosomes Us urinary space Glutaraldehyde 7000 \times

All nuclei were preserved No necrotic cells were observed Sometimes the urinary space especially near the tubular pole contained fragments of tubular epithelial cytoplasm evidently an artefact produced by the handling of the tissue before fixation

DISCUSSION

The glomeruli in this ultrastructural study of anuria were compared with those in 6 biopsies from patients without renal abnormalities In addition they were compared with the observations of the normal human glomerulus by Bergstrand & Bucht (1958) and with an exten

sive recent study of the normal fine structure of the human glomerulus by *Jorgensen* (1966)

Five patients with anuria described by *Dalgaard* (1960) did not show any glomerular lesions. The present study showed hyperplasia of epithelial cell cytoplasm in two and hyperplasia and proliferation of intercapillary (mesangial) cells with narrowing of the capillary lumina in one out of 12 biopsies. One patient described by *Regnier* (1959) had proliferation of the endothelial cells (intercapillary cells?). Perhaps these changes are to be regarded as cellular modifications connected with the sudden decrease of filtration. Since the other biopsies from patients with anuria did not show these changes they cannot be essential for production of the anuric state.

All biopsies from patients with anuria showed the presence of cytosomes in the epithelial cytoplasm and more rarely in the endothelial and intercapillary cell cytoplasm. These were not observed in the 5 control patients. Such cytosomes are presumed to be lysosomes and to contain hydrolytic enzymes. Their presence in cells where they do not ordinarily occur could be interpreted as a sign of augmented destruction of normal cellular components. This non specific type of damage was the only one which was seen in all the biopsies. None of the observed abnormalities in glomeruli seem to be of primary importance in the production of the lowered filtration which is presumed to occur in anuria.

SUMMARY

Twelve biopsies from ten patients with acute anuria were studied by electron microscopy. No severe lesions were observed. Two biopsies showed swelling of epithelial cytoplasm. In one case capillary lumina were occluded by swelling of endothelium and intercapillary cells. These three biopsies were all from early phases of anuria. In all other biopsies the glomeruli showed only slight non specific damage of the cells. The fine structure of the glomeruli in acute anuria cannot explain the lowered filtration rate which is presumed to occur in acute anuria.

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ELECTRON MICROSCOPIC AND CYTOCHEMICAL STUDIES OF ACID PHOSPHATASE AND ARYL SULFATASE DURING AUTOLYSIS

By

JAN L F ERICSSON PETER BIBERFELD & ROLF SELJELID

Received 30 ix 66

Autolysis implies autodigestion of cells and is generally considered to be operative in the deterioration of cell structure and function in the dead organisms. An increasing body of evidence (9, 12, 15, 18, 27, 32-34, 36, 37) indicates that different lytic enzymes—cathepsins and proteases, amino peptidases, sulfatases, acid phosphatase, and others—which are included in the group of lysosomal enzymes (8, 9) are located in specific cytoplasmic organelles, often termed "cytosomes" and "lysosomes" (18). It appears that during *in vivo* conditions all the lysosomal enzymes are enclosed in the cytosomes and do not have access to substrates in the surrounding cytoplasm (9). Although it has been suggested by some (6, 8, 10, 26, 40) that release of lysosomal enzymes from cytosomes may initiate the degenerative cellular alterations during autolysis and necrosis, unanimous agreement on this point has not been reached (37).

The introduction of methods for the fine structural demonstration of acid phosphatase appeared to aid in elucidating the role of lysosomal enzymes during different physiologic and pathologic alterations of cell function. However, the use of acid phosphatase as a lysosomal marker is hampered to some extent by the fact that it is presently not known whether all the lysosomal enzymes are present in all the single membrane limited bodies (cytosomes, lysosomes, etc.) known to contain acid phosphatase (9).

The possibility (22) of demonstrating a second lytic enzyme, splitting *p*-nitrocatechol sulfate (an aryl sulfatase) with its intracellular localization appears to be of great importance for reactions of lysosomal enzymes as a group. Thus, it is of great demon-

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stration of two lysosomal enzymes in the same organelle would add significantly to the interpretation of biochemical results and would support the theory of biochemical homogeneity of morphologically heterogeneous organelles.

In the present investigation we have studied the activity and localization of acid phosphatase and aryl sulfatase in hepatic parenchymal and renal proximal tubule cells during autolysis and compared the fine structural alterations with the cytochemical findings. Optimal and uniform preservation of fine structure has been secured by use of thin slices of tissue—approximately 0.5 mm thick—and comparisons of the results of fixation in a protein cross linking agent glutaraldehyde in addition to osmium tetroxide.

MATERIAL AND METHODS

Young male Sprague Dawley rats weighing 200–250 gms were sacrificed by decapitation following exsanguination and the carcasses were kept at room temperature (+20°C) for periods of 1, 2, 4, 8, 16, and 24 hours. At the appropriate time point the abdomens were opened and slices of liver and renal cortex approximately 0.5 mm thick were immersed for 3 hours in 3 per cent purified glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at +4°C. Tissues from normal rats fixed immediately after death served as controls and were treated in exactly the same manner as the ones from experimental animals. Following fixation tissues were washed at +4°C in 0.1 M tris maleate buffer (pH 7.4) containing 7.5 per cent sucrose for 24 hours.

For the demonstration of aryl sulfatase approximately 6 μ thick free floating frozen sections of glutaraldehyde fixed tissues were incubated in a medium prepared as follows: Thirty mg p nitrocatechol sulfate (Sigma) was dissolved in 50 ml veronal acetate buffer pH 5.4. Lead nitrate (0.16 ml of a 24 per cent solution) was added and the medium was adjusted to pH 5.4. A heavy precipitate which was formed when lead was added was removed by filtering the substrate solution. Incubations were performed at +37°C and incubation times were for renal cortex 30 minutes and for liver 60 minutes. Sections incubated in substrate free media served as controls. Following incubation sections were washed in 3 changes of 0.1 M acetate buffer pH 5.0 and were immersed briefly in dilute ammonium sulfide.

For the demonstration of acid phosphatase approximately 6 μ thick frozen sections were incubated in a Gomori type medium as previously described (17). Incubation times were for kidney 30 minutes and for liver 45 minutes. Sections incubated in appropriate substrates for aryl sulfatase and acid phosphatase and immersed in ammonium sulfide were mounted on glass slides in 1% celloidin gelatin. Some sections were stained with methyl green.

Tissues from normal liver and renal cortex and from liver and renal cortex subjected to a 24 hour period of "autolysis" were fixed for fine structural observations. Slices of tissue were processed for demonstration of acid phosphatase and aryl sulfatase as described above with the exception that the frozen sections were cut at 50 μ and the final step—immersion in ammonium sulfide—was avoided. The tissues were postfixated in osmium tetroxide (OsO_4) and processed for electron microscopy as indicated below.

For the fine structural studies of exclusively OsO_4 fixed tissue small cubes of liver and outer renal cortex were immersed in 2 per cent OsO_4 buffered with acollidine (pH 7.4) for 1½–2 hours at +4°C. Dehydration and embedding in Epon was performed as previously described (15, 18). Thin sections were cut on LKB Ultratomes and were stained with lead citrate and uranyl acetate or a combination of lead hydroxide and uranyl acetate. They were studied in a Siemens Elmiskop I electron microscope. Thicker sections cut at 1 μ of Epon embedded tissues were stained with toluidine blue and were studied in the light microscope. When blocks of osmium fixed tissues were studied the specimens were trimmed in such a way that only the peripheral portions were included for thin sectioning.

RESULTS

I *Gross and Light Microscopy*

Gross Livers and kidneys appeared moderately enlarged and swollen in animals left in room temperature for 24 hours. The cut surfaces were pale at this time and the tissues were soft and friable. Similar although less pronounced alterations were noted at earlier intervals after sacrifice and were first noticeable at 4 hours.

Light microscopy In sections of glutaraldehyde-fixed hepatic parenchymal and renal cortical cells embedded in paraffin, very slight alterations were noted at 24 hours. These consisted in cytoplasmic swelling and an increased prominence of cytoplasmic "granules" presumed to represent mitochondria. In the cells of the proximal and distal convoluted tubules, the normally slender and elongated mitochondria seemed to become shorter and take on a more rounded shape. This alteration was more clearly revealed in sections of Epon-embedded tissues stained with toluidine blue (29). All the proximal tubules appeared collapsed with obliterated lumens.

II *Histochemistry*A *Liver*

1 **Acid phosphatase** In hepatic parenchymal cells of control animals activity was confined to small droplets (up to 1μ in diameter) which usually showed the typical pericanalicular arrangement (Fig. 1). No alteration in the quantity, appearance, or distribution of these droplets was noted at any interval up to 24 hours (Fig. 2).

2 **Aryl sulfatase** As was noted with acid phosphatase, reaction product in normal hepatic parenchymal cells was confined to cytoplasmic droplets with a largest diameter of approximately 1μ . With the incubation time employed (1 hour), the number of these droplets appeared to be considerably lower than that of acid phosphatase droplets. The aryl sulfatase droplets showed the same localization as the acid phosphatase positive droplets, however, and were mainly confined to peribiliary regions. The appearance, quantity, and intracellular distribution of aryl sulfatase droplets at 24 hours (Fig. 7) was inseparable from that of controls.

B *Proximal Convoluted Tubules*

1 **Acid phosphatase** In control specimens, the reaction product was precipitated in cytoplasmic droplets (15-18) (see Fig. 3). No alterations were seen in experimental animals (compare Fig. 3 with Fig. 4).

2 **Aryl sulfatase** Reactive sites were in the form of droplets with a size and distribution corresponding to that of the acid phosphatase droplets (see Fig. 5). In addition, moderate numbers of very small droplets ($0.2-0.4\mu$ in diameter) were observed in some proximal tubu-

stration of two lysosomal enzymes in the same organelle would add significantly to the interpretation of biochemical results and would support the theory of biochemical homogeneity of morphologically heterogeneous organelles.

In the present investigation we have studied the activity and localization of acid phosphatase and aryl sulfatase in hepatic parenchymal and renal proximal tubule cells during autolysis and compared the fine structural alterations with the cytochemical findings. Optimal and uniform preservation of fine structure has been secured by use of thin slices of tissue—approximately 0.5 mm thick—and comparisons of the results of fixation in a protein cross linking agent glutaraldehyde in addition to osmium tetroxide.

MATERIAL AND METHODS

Young male Sprague Dawley rats weighing 200–250 gms were sacrificed by decapitation following exsanguination and the carcasses were kept at room temperature (+20°C) for periods of 1, 2, 4, 8, 16 and 24 hours. At the appropriate time point the abdomens were opened and slices of liver and renal cortex approximately 0.5 mm thick were immersed for 3 hours in 3 per cent purified glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at +4°C. Tissues from normal rats fixed immediately after death served as controls and were treated in exactly the same manner as the ones from experimental animals. Following fixation tissues were washed at +4°C in 0.1 M tris maleate buffer (pH 7.4) containing 7.5 per cent sucrose for 49–24 hours.

For the demonstration of aryl sulfatase approximately 6 μ thick free floating frozen sections of glutaraldehyde fixed tissues were incubated in a medium prepared as follows. Thirty mg π nitrocatechol sulfate (Sigma) was dissolved in 50 ml veronal acetate buffer pH 5.4. Lead nitrate (0.16 ml of a 24 per cent solution) was added and the medium was adjusted to pH 5.4. A heavy precipitate which was formed when lead was added was removed by filtering the substrate solution. Incubations were performed at +37°C and incubation times were for renal cortex 30 minutes and for liver 60 minutes. Sections incubated in substrate free media served as controls. Following incubation sections were washed in 3 changes of 0.1 M acetate buffer pH 5.0 and were immersed briefly in dilute ammonium sulfide.

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lar cells. At 24 hours the distribution and activity of aryl sulfatase appeared to be similar to that in controls.

Sections from control and experimental animals incubated in media lacking the substrates (*β* glycerophosphate or *p* nitro catechol sulfate) lacked evidence of precipitation of reaction product.

III Electron Microscopy

A Controls

The appearance of hepatic parenchymal and renal proximal tubule cells in control animals was in accordance with previous observations (7, 12, 15-18, 28, 36-39).

B Experimental Animals

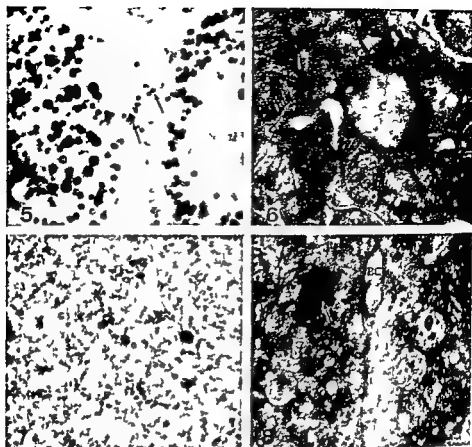
1 Hepatic parenchymal cells Within the cytoplasm fine structural alterations were noted in mitochondria, microbodies, endoplasmic reticulum, and cytoplasmic ground substance (Fig. 9). These alterations were diffusely distributed within the cells. They were of similar appearance in osmium fixed and glutaraldehyde fixed tissues. Many mitochondria appeared more rounded than in controls and all of them lacked matrical granules. The matrix was of uniform density and alterations of cristae or the envelope were only rarely encountered. The rough surfaced endoplasmic reticulum showed irregular widening of the cisternae which sometimes revealed focal loss of ribosomes. Polyosomes were not encountered. Smooth surfaced endoplasmic reticulum tended to break up into vesicular elements. Microbodies showed pallor of their matrix but otherwise appeared normal. The cytoplasmic matrix showed focal areas of pallor and appeared to be more voluminous than in controls with cytoplasmic organelles widely separated from one another.

Contrasting to these changes was the unaltered appearance of cytosomes (Fig. 10). Although occasional cytesegresomes (autophagic vacuoles) were observed these were not more frequent than in controls.

2 Proximal convoluted tubules For a description of the fine struc-

Figs 1-5

Fig 1	Liver control animal	Discrete droplets ma	1	2	... ty of
	bile capill es X				
Fig 2	Liver 24 hours after sacrifice	Droplets of sim l r app	an	2	Fig 1
	are present in peri	alicular areas X 75			
Fig 3	Kidney control animal	Abundant large vacu	1	2	convoluted
	tubules & glomeruli	contains a few small r	ti	40	
Fig 4	Kidney 24 hours after sacrifice	Acid phosphat e ac	le	1	similar to the
	ones shown in Fig 3	present in proximal cor	oute	bul s X 750	



Figs 5-8

- Fig 5** kidney control animal Section incubated for the demonstration of aryl sulfatase Activity in proximal convoluted tubules is in the form of large droplets similar to the ones in the acid phosphatase procedure Note the small droplets (arrows) which probably are located in distal tubules or collecting ducts $\times 800$
- Fig 6** kidney control animal Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing localization of final product along the margins of cytosome (C) in a proximal convoluted tubule cell $\times 11,400$
- Fig 7** Liver 24 hours after sacrifice Section incubated for the demonstration of aryl sulfatase Activity is confined to small discrete droplets which are less numerous than the acid phosphatase droplets (cf Fig 2) but appear to show the same localization around bile capillaries $\times 530$
- Fig 8** Liver 24 hours after sacrifice Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing a cytosome (C) with final product precipitated along the periphery Note absence of reaction product in two microbodies (Mb) BC bile capillary $\times 11,000$

ture of the normal proximal tubule cells see previous publications (16-18)

In the experimental animals alterations of mitochondria were more prominent than in hepatic parenchymal cells (Fig 11) Thus the tendency to attain rounded forms (29) was more apparent and alterations

of the matrix substance with focal pallor were evident. Matrical granules were not observed. The cristae were numerous. Often long cristae connected one side of the envelope with the other. Such cristae appeared to be much longer than in the controls. While dilatation and fragmentation of the endoplasmic reticulum was frequently observed, increased area of cytoplasmic ground substance was not in evidence. As in hepatic parenchymal cells, cytosomes appeared unaltered.

IV Ultrastructural Cytochemistry

A Acid Phosphatase

In normal hepatic parenchymal and proximal tubule cells the reaction product is localized in cytosomes, cytosegresomes and on occasional Golgi membranes (15-18). In accordance with the light microscopic observations a similar localization was noted in the experimental animals in the present study.

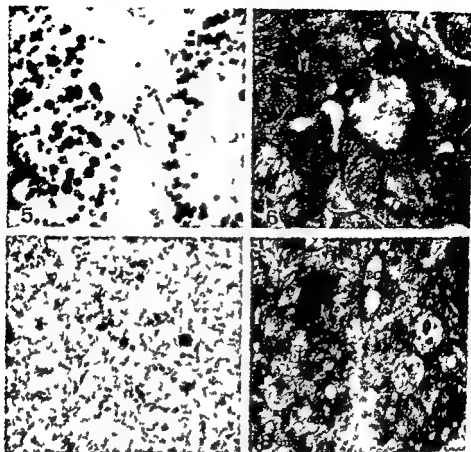
B Aryl Sulfatase

In the cells of the proximal tubules of *control animals* reaction product was precipitated in the cytosomes (Fig. 6). Often the final product appeared as a narrow rim of precipitate overlying the membrane limiting the cytosomes. Although occasional cytosomes lacked reaction product, precipitation in relation to cytosomes appeared to be the rule. Observations are presently too few to permit definitive conclusions concerning the possible occurrence of aryl sulfatase in the Golgi apparatus and cytosegresomes. Likewise the nature of the very small reactive sites seen in the light microscope is not clear. Microbodies did not seem to contain final product. In hepatic parenchymal cells some cytosomes contained reaction product.

As demonstrated in Fig. 8 reaction product was confined to cytosomes and cytosegresome-like bodies in the *experimental animals*. Spreading of reaction product was not observed.

DISCUSSION

At the present state of our knowledge it is most convenient to base a definition of cellular death upon the notion of irreversibility (26). The time of cell death can accordingly be identified with the time at which the point of no return is reached. In the present simple system for autolysis cellular death is probably the result of the combined effects of anoxia and lack of exogenous and endogenous substrates. Studies of ischemia by various authors (2, 5, 26) have indicated that hepatic parenchymal cells can tolerate 30 to 40 minutes of total ischemia without reaching the point of no return. The alterations observed 24 hours after sacrifice of the experimental animal apparently represent alterations far beyond those at the point of death and are representative of autolysis.



Figures 5-8

- Fig 5** kidney control animal Section incubated for the demonstration of aryl sulfatase Activity in proximal convoluted tubules is in the form of large droplets similar to the ones in the acid phosphatase procedure Note the small droplets (arrows) which probably are located in distal tubules or collecting ducts $\times 800$
- Fig 6** kidney control animal Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing localization of final product along the margins of cytochrome (C) in a proximal convoluted tubule cell $\times 19,400$
- Fig 7** Liver 24 hours after sacrifice Section incubated for the demonstration of aryl sulfatase Activity is confined to small discrete droplets which are less numerous than the acid phosphatase droplets (cf Fig 2) but appear to show the same localization around bile capillaries $\times 580$
- Fig 8** Liver 24 hours after sacrifice Section incubated for the fine structural demonstration of aryl sulfatase Electron micrograph showing a cytochrome (C) with final product precipitated along the periphery Note absence of reaction product in two microbodies (Mb) and bile capillaries $\times 18,000$

ture of the normal proximal tubule cells see previous publications (14, 16-18)

In the experimental animals alterations of mitochondria were more prominent than in hepatic parenchymal cells (Fig 11) Thus the tendency to attain rounded forms (29) was more apparent and alterations

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THREE DANISH CASES OF MALIGNANT CHILDHOOD LYMPHOMA (BURKITT)

By

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Received 29 IX 66

In 1968 *Burkitt* pointed out that a characteristic jaw sarcoma often seen in children in Uganda where it originally had been described in 1961 by Cook was part of a multifocal tumour syndrome affecting abdominal viscera. *O'Connor & Davies* added the information that jaw and abdominal tumours of identical lymphomatous structure constituted about half of all malignant childhood tumours registered in the Kampala Cancer Registry Uganda.

When in 1961 *O'Connor* reviewed 106 cases including 28 examined post mortem he found the African lymphoma a pathological entity but classified the vast majority or 83 per cent of cases as a poorly differentiated malignant lymphoma of lymphocytic type while the rest were designated as stem cell histiocytic and mixed lymphocytic histiocytic lymphomas. *Hull & Wright* (1963) however drew the limits somewhat more narrowly. They found the presence of multifocal jaw tumours in a child the most certain clinical criterion and the combination of a single jaw tumour with tumour in the thyroid kidneys or ovaries or extradural spinal deposits causing paraplegia they considered virtually diagnostic of this tumour syndrome. However they found that all cases should be biopsied and that only those conforming to defined histological criteria should be included. Histologically they found that the tumour always is a poorly differentiated lymphocytic (lymphoblastic) lymphoma containing a variable admixture of non malignant histiocytes.

Wright (1963) on the basis of the Kampala material described the tumours as composed of sheets of round cells with round or uniform nuclei a well defined nuclear membrane and one to five often in conspicuous eosinophilic nucleoli with a narrow rim of amphophilic cytoplasm. Interspersed between the lymphoid cells are numerous histiocytes usually with abundant clear or vacuolated cytoplasm and often actively phagocytic containing large amounts of degenerated nuclear debris. These cells do not have the characteristic features of malignant cells and give the histological picture a characteristic berry like feature which however is not constant. Its prevalence as *Wright* varies

with different fixation, sectioning, and staining procedures and with the type of tissue being invaded. Post mortem loosening and separation of cells lessens its prominence in certain tissues.

Wright (1964) reports that occasionally leukaemia occurs as a terminal manifestation of the disease and it seems interesting that while leukaemia in Denmark amounts to about half of all malignant diseases in childhood (Clemmensen 1965) it is very rare in Africa (Davies 1958). It follows from local conditions that estimates of incidence, not to speak of age distribution, will be approximate only in those regions but Burlitt (1963) on the basis of 144 cases estimated incidence of the childhood lymphoma in Uganda at 1 per 50-100 000 with local findings ranging up to 8 among a population of about 100 000. From case finding studies he found the disease limited to a belt across Africa limited in the North by the Sahara and towards the South by a border line through Angola traversing the southern tip of Congo, excluding Zaire and Rhodesia but including Tanzania and Malawi. According to Burlitt the lymphosarcoma is extremely rare in the highlands of Kenya and Ruanda, Burundi and he suggests that the distribution of the tumour depends on humidity and vegetation explainable by the further assumption of an arthropod vector. Apparently the absence of the syndrome in the Kinshasa (Leopoldville) area is out of harmony with local humidity and vegetation but is explained as due to an energetic extermination of mosquitoes in that area.

Cases resembling those published from Central Africa have during later years been published from various parts of the world. From South Africa Glucksman (1963) reported four cases in white and four in non white children and single cases as well as series have been published from the United Kingdom (Wright 1964, the United States O'Connor et al 1965, Dorfman 1965) from Papua and New Guinea (ten Seldam et al 1966) and Columbia, South America (Beltram 1966). Up to now no cases seem to have been reported from the European continent and since a recent case in this institute turned our attention to the likelihood of similar cases records were reviewed for autopsy cases presenting the typical histologic features.

CASES

Among post mortems from the years 1955 to Aug. 1966 representing a total of 1615 autopsies 36 had been performed on persons aged below 20 years and suffering from diseases of the lymphatic and reticular system. Besides the recent case of a boy diagnosed from biopsy two autopsy records for girls showed the histologic features characteristic of Burlitt's syndrome.

Case 1

Thirteen year old daughter of litter. For a couple of years repeated catarrhal infections treated with tonsillectomy. For two months before admission swelling in

TABLE 1
Clinical Findings

Case No	Sex	Age	Site of primary	Peripheral lymph nodes	White cell count	Total duration of illness	Treatment
1 167/59	♀	13	Right parotid	not involved	3700	4 months	Operation & X rays
2 15/66	♀	9	Extra dural spinal canal	not involved	7300	1 month	Operation
3 72/66	♂	4	Left lat cerv reg	not involved	5900	2 months	X rays

TABLE 2
Involvement of Organs in 3 Autopsy Cases

	Case 1	Case 2	Case 3
Skin	—	—	—
Tonsils	—	—	+
Neck	—	+	+
Mediastinal Glands	+	+	+
Retroperitoneal lymph glands	—	+	+
Lungs	—	+	+
Heart	+	—	—
Stomach	+	+	+
Small Intestine	+	—	—
Bowel & Rectum	—	—	—
Liver	—	—	—
Spleen	—	—	+
Pancreas	+	+	—
Supra Renal Glands	—	+	—
Kidney	+	—	+
Ovaries/Testes	+	+	—
Bone Marrow	+	+	+
Spinal Canal	—	+	—
Thyroid	—	+	—

the right side of the neck, rapidly increasing during the last week. On admission a tumour of the right parotid gland measuring ca $3 \times 4 \times 5$ cm. Differential count normal. Totoplasmosis sero reaction negative. Partial removal of tumour was followed by application of 3330 R. Death six weeks after admission.

Post mortem. Mass of right parotid region. Enlargement of lymph nodes of mediastinum measuring up to 2×3 cm and nodular infiltrations of pericardium, gastric and intestinal mucosa of up to 2 cm size. Ovaries measured $9 \times 4 \times 1$ cm and pancreas and kidneys showed considerable infiltration with enlargement of spleen and bone marrow showed histologically demonstrable infiltration.

Case 2

Nine year old daughter of cabinet maker. Admitted by sudden development of mellary transection syndrome. Age 3 1/2 years. Responding to the level of 4th to 9th thoracic vertebra was followed by the patient died two weeks later.

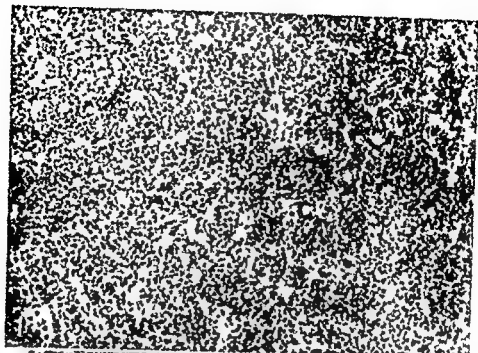


Fig 1 Case 1 Primary tumor in parotis (H + E, $\times 80$)

Fig 2 Case 2 Tumor infiltration in pancreas with preservation of "starry sky" appearance (H + E, $\times 80$)

Post mortem showed lymphosarcoma of the thymus and medulla, large epidural with secondary myelomalacia (Histologist Ernst Christensen). Lymph nodes of neck, mediastinum and along the abdominal aorta were enlarged measuring up to 7 cm. Extensive infiltration of tumor tissue was found in the thyroid, the

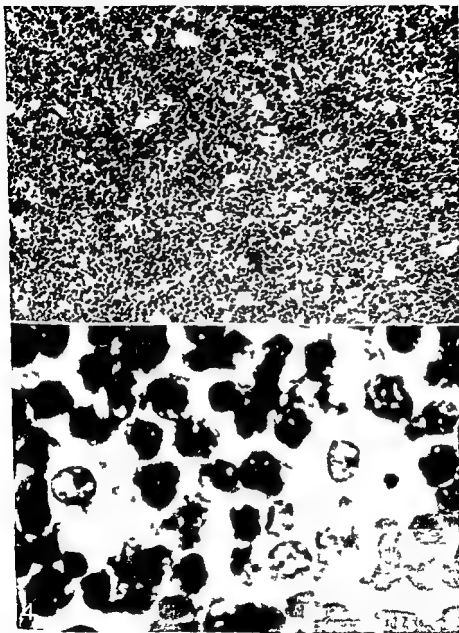


Fig 3 Case 3 Primary tumor in lymph node in right cortex (H + E \times 80)

Fig 4 Case 3 Detail of Fig 3 showing phagocytosis of the pale cells and abundant ill defined cytoplasm. The scattering of the cells in the tissue constitutes the 'starry sky' appearance (H + E \times 800)

lungs pancreas and surroundings and adrenals. Gastric mucosa showed nodular infiltrations ovaries measured $8 \times 4 \times 2$ cm infiltrated with tumour tissue. There was gross infiltration of bone marrow and microscopic infiltration of the spleen. No visible enlargement of axillary and inguinal lymph nodes.

Case 3

Four year old son of water and gasfitter. The family never left Denmark except for the mother's visit to Italy some six months before the birth of the patient. Six months before admission the father and the son developed "influenza". One month before admission a node was noticed on the left side of the neck persisting in spite of treatment with penicillin and measuring $7 \times 4 \times 5$ cm located with its upper pole levelling with the mandibular angle and its anterior limit coinciding with that of the sternocleid muscle. A soft microadenitis of the accessory chain was noticeable but no other nodes discernable. Totoplasmosis sero reaction negative. After treatment with 1075 II the patient died two weeks after admission.

Post mortem examination showed the enlarged lymph nodes in the neck as described. Further enlarged nodes along the aorta and in the mesenteries measured up to 2×3 cm. Infiltrates of tumour tissue were found in the columna and in both kidneys.

A survey of clinical and pathological findings is given in Tables 1 and 2.

The histological findings of the three cases were all alike and in full correspondence with the description given by Wright. Within the limitations of practical pathology the cases must then be considered as indistinguishable from those described as Burkitt's tumour. It is true that in Africa the disease is rarely reported to have been observed first in one of the palpable lymph nodes like in our case 3 but this can hardly be considered a decisive criterion.

The histological pictures have been illustrated by photomicrographs 1-4.

It remains an open question to which extent the demonstration of scattered reticulum cells in a childhood lymphosarcoma—the so-called "starry sky" appearance will entitle us to assume a clinical entity but it does seem more likely than not that the post mortem findings in the present three cases and perhaps also their lack of radiosensitivity will designate them as belonging to the group of childhood lymphosarcomas often termed Burkitt's tumour.

SUMMARY

Report on three cases of childhood lymphosarcoma with histologic and radiologic resemblance to Burkitt's African lymphosarcoma in children diagnosed in Denmark.

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AN IMPROVED METHOD FOR THE HISTOCHEMICAL DEMONSTRATION OF PHOSPHORYLASE IN TISSUE SECTIONS

By

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The principles for the histochemical demonstration of phosphorylase activity in tissue sections were first established by *Talbot & Kurachi* (4). Unfixed cryostat sections were incubated in a medium containing glucose 1 phosphate and the co factor muscle adenylic acid. Tissue components possessing sufficient phosphorylase activity synthesised iodophilic polysaccharides from glucose 1 phosphate and after incubation the sites of phosphorylase activity were visualized by simple iodine staining.

The sensitivity and the exactness of localization in enzymatic histochemical procedures are fully depend on the tendency of the enzyme and the reaction product to diffuse during incubation and in the case of the phosphorylase reaction the conditions are particularly unfavourable: the enzyme is localized in the ground substance of the cytoplasm and the reaction product is a water soluble polysaccharide. To diminish this difficulty *Talbot* recommended the inclusion of ethanol (20 per cent v/v) in the incubating medium (3, 4) and later *Frank & Pallania* advocated the additional inclusion of polyvinyl pyrrolidone (PVP) (7.5 per cent w/v) (1). We found however that these improvements did not give satisfactory demonstration of phosphorylase particularly in gastrointestinal epithelium and it was therefore decided to determine the optimum concentrations of protective agents in the phosphorylase medium.

MATERIAL AND METHODS

Biopsies of human tissues were taken during endoscopy (rectal mucosa) percutaneously (liver) or during operations (skin, striated muscle, lonic mucosa, colon and rectal tumours). The biopsies were placed on thin disc of cork and quenched by immersion in liquid nitrogen. Sections were cut at 8-10 μ using a SL 1 cryostat. All incubating media described below were tested on nine rectal biopsies (from patients suffering from irritable colon or ulcerative colitis), one rectal polyp, one liver biopsy, four skin biopsies and four muscle biopsies. Incubating medium C (see below) was tested on a greater number of tissue specimens.

Incubating media. Nine different incubating media (labelled A to I) were prepared, all containing 50 mg of glucose 1 phosphate, 5 mg of muscle adenylic acid, 1 mg of

glycogen and 9 mg of sodium fluoride in 5 ml of 0.1 M acetate buffer (pH 6.0). The concentrations are those recommended by Erdős & Palkama (1). To media A, B and C were added no ethanol and 0.05 and 1.0 g of polyvinyl pyrrolidone (PVP) (average MW = 17000 obtained from Koch Light Ltd) respectively. To media D, E and F were added 0.5 ml of ethanol and 0.05 and 1.0 g of PVP respectively. To media G, H and I were added 1.0 ml of ethanol and 0.05 and 1.0 g of PVP respectively (Table 1). When stored at 4°C the media were stable for at least one week. The media were preheated to room temperature before use.



Fig. 1

Histochemical procedure 1) Mounting of frozen sections on slides and air drying for 5 minutes. 2) Incubation for one hour at 37°C under conditions suitable to prevent evaporation. For this purpose a slide with a central shallow concavity was used. The concavity was filled with incubating medium and the slide with the section was placed on top of the concavity thus forming a closed incubation chamber (Fig. 1). Air bubbles were avoided. 3) Removal of slide with section and drying in air. 4) Rinsing in 40 per cent ethanol for 2 minutes. 5) Air drying. 6) Fixation in absolute ethanol for 5 minutes. 7) Air drying. 8) Iodine staining. One or two drops of glycerol iodine (equal parts of Gram's iodine solution and glycerol) were placed on the section. After a few minutes a coverslip was mounted on top. The iodine staining was stable for several hours.

Control experiments Sections were treated with an incubating medium from which glucose 1-phosphate had been omitted.

Evaluation The staining intensity of the various tissue components in the preparations were roughly graded according to an arbitrary scale ranging from (+) to ++++. The preparations were coded so that it was not known during microscopy of a preparation which incubating medium had been used.

RESULTS

The polysaccharides which were synthesised in the tissue sections during incubation stained violet or blue with iodine. The staining intensity was recorded and the more important results are summarized in Table 1. The content of protective agents in the incubating medium greatly influenced the outcome of the phosphorylase reaction. Phosphorylase activity was only demonstrated satisfactorily in rectal epithelium when the incubating medium had a high concentration of PVP (media C and F) whereas an additional high concentration of ethanol inhibited the reaction (medium I). When medium G was used the surface and crypt epithelium presented a strong staining (Fig. 2c and d). All other media gave unsatisfactory results including G and H which as recommended in previous works (1, 3, 4) had a high ethanol concentration but contained little or no PVP. Using those media the sections were either unstained or presented weak staining as depicted in Fig. 2a and b.

It was also noticeable that sections incubated in media with a high PVP content were very well preserved whereas the other media damaged the tissue during incubation to a varying degree.

A high PVP content also improved the phosphorylase reaction in

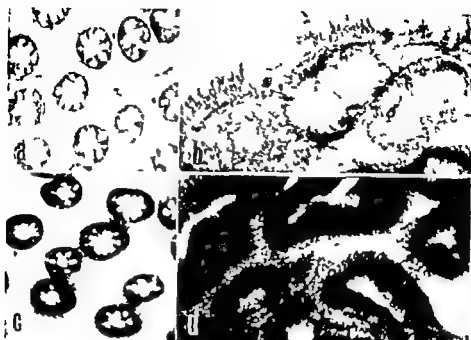


Fig. 7

a and b—Weak staining reactions in crypts of Heterkühn and a rectal p. lyp after incubation in phosphorylase media C and H. c and d—Intense staining reactions in the same structures after incubation in phosphorylase medium C.

TABLE I

The Staining Intensity Obtained Using Incubating Media with Different Ethanol and Polyvinyl Pyrrolidone (PVP) Contents Has Been Graded According to an Arbitrary Scale

Incubating medium			Staining intensity			
Medium	Ethanol (ml added to ml medium)	PVP (g added to 5 ml medium)	Rectal mucosa			
			Crypts of Heterkühn	Mucularis mucosae	Epithelium	Striated muscle
A	0	0	(+)	(+)	+	+++
B	0	0.5	+	+	+	++++
C	0	1.0	+++	+++	++	++++
D	0.5	0	(+)	(+)	+	+++
E	0.5	0.5	++	+++	+	++++
F	0.5	1.0	+++	+++	++	++++
G	1.0	0	(+)	(+)	+	+++
H	1.0	0.5	+	++	+	+++
I	1.0	1.0	+	++	+	+++

(+) weak or no staining + weak staining ++ moderate staining
+++ strong staining ++++ intense staining

smooth muscle epidermis and liver cells whereas satisfactory staining of striated muscle was obtained using any of the media

To test the reproducibility of the satisfactory results which were obtained using incubating medium C a greater number of tissue specimens were examined using this medium only In all cases the results were comparable to those described

Control preparations incubated in media without glucose 1 phosphate showed no staining with iodine

DISCUSSION

In the histochemical experiment phosphorylase in conjunction with various branching factors catalyzes the synthesis of iodophilic polysaccharides from glucose 1 phosphate whereas phosphorylase *in vivo* is mainly concerned with the opposite process *i.e.* degradation of glycogen to glucose 1 phosphate (2) The enzyme thus plays an important role in glycogen metabolism

The present experiments show that the histochemical demonstration of phosphorylase in some tissue structures may be greatly improved by increasing the viscosity of the incubating medium through the addition of a larger quantity of PVP (200 mg per ml) This modification resulted in stronger staining reactions in a number of tissue structures presumably by reducing the diffusion of polysaccharides into the medium and at the same time it prevented damage to the tissue sections during incubation The modification also permitted the use of thin sections (8-10 μ) instead of sections of 40 μ as recommended by Takeuchi & Kuriaki (4) Addition of ethanol to the medium was not found to be advantageous in these experiments but is essential for the detection of phosphorylase in blood films (5)

It is envisaged that the use of high concentrations of PVP may also prove of value in other histochemical enzyme reactions

SUMMARY

The histochemical demonstration of phosphorylase in tissue sections is hampered by the fact that the end product of the reaction is water soluble If however the viscosity of the incubating medium is increased by addition of a larger quantity of polyvinyl pyrrolidone than usually recommended the demonstration of the enzyme is greatly facilitated Additional inclusion of ethanol in the medium only increases the reaction This modification is particularly valuable for the demonstration of phosphorylase in renal epithelium

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Control mice Ovaries of untreated control mice of the same strain and age were examined and compared with the ovaries of mice in the 3 experimental groups.

All mice were weaned at the age of 4 ½ weeks and housed 2-5 mice in a cage until sacrifice. Mice were killed and their ovaries examined at different time intervals after DMBA treatment. The ovaries were fixed in Bouin's solution, imbedded in paraffine, serially sectioned at 5 μ and stained in haematoxylin-eosin. The pathology of the ovaries was studied and differential counts of the oocytes were done (12). The oocytes were divided into two main groups: 1) The small oocytes i.e. 20 μ or less in diameter; 2) The growing and large oocyte i.e. those germ cells that have begun to grow and have a diameter of more than 20 μ as well as those that have reached their maximum size of 100 μ (13).

RESULTS

Oocyte Counts

The oocyte counts of all three experimental groups are given in Table 1. The numbers of oocytes counted in the ovaries of DMBA treated mice were expressed as a percentage of oocytes in the ovaries of not treated control mice of the same age.

TABLE 1

Surviving Oocytes at Different Ages after Treatment with DMBA by Different Routes

	Group I (Street) (direct ovary painting)		Group II (Bagg) (feeding)		Group III (Bagg) (intraperitoneal injection)	
	Small oocytes %	Growing and large oocytes %	Small oocytes %	Growing and large oocytes %	Small oocytes %	Growing and large oocytes %
14 days			63	100	15	49
30 days	57	97				
47 days			4	58	0	17
49 days	33	77				
3 months	6	28	0.7	10	0	0
6 months	0.2	1.4	0	0	0	0
9 months	0	0.5				
12 months	0	0				

Group I (direct ovary painting) In the ovaries examined 14 days after the organs were painted with DMBA, almost one half of the small oocytes were destroyed. At the age of 49 days (28 days after painting) only one third of the small oocytes survived. At the age of 6 months the ovaries contained between 0 and 7 small oocytes. A normal 6 months old Street mouse had 900 small oocytes in one ovary. After the age of 6 months no small oocytes survived in the DMBA painted ovaries.

The growing and large oocytes were also destroyed but at a slower rate. Ovaries of 4 mice were examined at the age of 9 months. These contained between 0 and 1 growing and large oocytes. A normal Street mouse aged 9 months had 300 growing and large oocytes in one ovary.

Group II (feeding) The reduction in the number of oocytes in this group occurred faster than in the previous one. At the age of 42 days (21 days after feeding of DMBA) only 4 per cent of the small oocytes survived. In group I the same proportion of surviving small oocytes was not reached until the age of 3 months i.e. 70 days after DMBA-painting of the ovaries. In group II small oocytes were not observed after the age of 3 months. As in group I the growing and large oocytes were destroyed at a lower rate than the small ones at the age of 42 days about one half survived at 3 months 10 per cent were still found however after this age none were found any more.

Group III (intraperitoneal injection) A very rapid destruction of oocytes occurred in this group. Already 7 days after intraperitoneal injection of DMBA (age 28 days) only 15 per cent of the normal complement of small oocytes were still present. At the age of 42 days (21 days after injection) the ovaries were depleted of small oocytes while about one fifth of the growing and large oocytes survived. A normal Bagb mouse aged 42 days had 5000 small and 320 growing and large oocytes in one ovary. At the age of 3 months no more oocytes were present in the ovaries of the DMBA injected mice.

Pathology

The histologic changes in the first 2 months after treatment with DMBA were insignificant except for the reduction of oocytes in all stages of follicle development.

Group I (direct ovary painting) At the age of 3 months the histologic structure of the ovaries was still dominated by follicles in different stages of development although their number was reduced. Compared with the control ovaries however a larger number of degenerated follicles was noted and the number of old corpora lutea was increased. The amount of stroma was markedly increased, this was luteinized and formed groups and cords throughout the ovaries. Rings of follicle cells without oocytes (empty rings) formed a rim just beneath the surface epithelium in one of the ovaries examined.

At the age of 6 months the histologic picture was no longer dominated by developing and ripe follicles. Just beneath the surface epithelium which showed a few downgrowths a rim of empty rings and small solid balls of follicle cells surrounded by a layer of flattened theca cells (pseudo follicles) was seen. Some degenerated follicles were present but an accumulation of corpora lutea, some of which showed a tendency of merging, occupied most of the organ. The stroma was luteinized.

Unilateral non infiltrating ovarian tumours were found in two mice out of four at the age of 8 months. These were luteomas, they consisted of nodules of lutein cells. The ovaries of mice that had not developed tumours at this age showed the same pathologic picture as seen at 6 months.

4 mice were examined at the age of 12 months. They had all unilateral ovarian tumours. 3 were luteomas showing growth into the periovarian fat tissue while one was a benign granulosa cell tumour.

Group II (feeding) At the age of 3 months the cortex of the ovaries showed empty rings and pseudo follicles as well as a few normal and degenerating follicle. The center of the ovaries was mainly composed of well defined or merging corpora lutea (in one single section as many as 14 different corpora lutea could be counted) and central cords of luteinized stroma.

At the age of 6 months two mice out of two had a unilateral granulosa cell ovarian tumour.

Group III (intraperitoneal injection) At the age of 3 months the ovary contained only few empty rings and pseudo follicles, no follicles normal or degenerating were found. Almost the entire organ was composed of merging corpora lutea i.e. one mass of corpus luteum material in which separate corpora lutea could not be identified. Stroma was not present.

At the age of 6 months unilateral granulosa cell tumours were found in two out of two mice. Neither oocytes nor rests of follicles or corpora lutea or any other constituent of a normal ovary were recognized in these ovarian tumours.

DISCUSSION

Tumour Incidence

The number of experimental animals in the present material is too small for statistical evaluation. Nevertheless the results show an early development and a high incidence of ovarian tumours. These results were obtained using only a single application and a smaller total dose of DMBA than has been used before. In *group I* the ovaries were puncted directly with a 0.5 per cent solution of DMBA. Though the absolute dose of DMBA that was applied cannot be calculated it is presumably less than 0.2 mg of DMBA. In this group half of the animals had developed ovarian tumours at the age of 9 months and all the animals had ovarian tumours at the age of 12 months. In *group II* 0.2 mg DMBA was fed to the animals and both mice at the age of 6 months had ovarian tumours. In *group III* 0.2 mg of DMBA was injected intraperitoneally to the animals and also in this group ovarian tumours were already found in both mice aged 6 months.

Marchant (11) puncted the skin of mice aged 2½ to 4 months 6 times at fortnightly intervals, the total dose of DMBA being 6 mg. 7 months after first skinpuncturing she found 70 per cent of the mice with predominantly unilateral ovarian tumours.

Blanchfort et al. (1) fed 2½ to 4 months old mice 8 times at weekly intervals, the total dose of DMBA being 8 mg. During the entire observation period, which extended over 9 months 68 per cent of the mice had developed ovarian tumours.

In his investigations on the development of spontaneous tumours in mice *Leferre* (9) found a maximum of 27 per cent ovarian tumours in 9 to 11 months old mice. In that experiment mice aged 1½ months were skinpainted once weekly for 24 weeks. The total dose of DMBA has not been reported.

The high tumour incidence in the present experiment may be explained in three different ways.

First the strains of mice used by the author (*Street & Bagg*) may be especially susceptible to the development of ovarian tumours after treatment with DMBA. It is known that different strains of mice have a different sensitivity to tumour development after treatment with carcinogenic chemicals (1-4).

Second the routes of appl. in., the DMBA may be of importance for the development and the incidence of the induced tumours. Direct ovary painting with DMBA followed by development of ovarian tumours has not been reported earlier. *Leferre* (9) found 2 ovarian carcinomas in mice aged 8 months following intraperitoneal injection of DMBA, but the observation was not followed up and the age of the mice at the time of injection and the dose of DMBA used is not reported.

It could be expected that direct ovary painting or intraperitoneal injection of DMBA would result in a higher tumour incidence than other routes of application as the carcinogen in those cases comes into more intimate contact with the ovaries. It could also be expected that feeding the DMBA would result in a higher tumour incidence than painting the skin as the DMBA in the first case is applied internally without any risk of being lost from the organism. Whether the carcinogen after skinpainting is mainly absorbed through the skin or whether it is mainly licked off and consumed is not known but once applied on the external hairy surface some of the DMBA may be lost.

However it is not likely that the different routes of carcinogen application alone are responsible for different incidences of ovarian tumour development for the following reasons. In the skinpainting experiments (11) the total dose of carcinogen were so much larger (6 mg of DMBA) than in the present study (0.25 mg) that the ovaries in spite of the indirect route of application probably have been exposed to at least the same amount of DMBA as after direct ovary painting and intraperitoneal injection. Furthermore if the present feeding experiment (group II) is compared with the feeding experiment of *Bianchi-Sforzi et al* (1) it is seen that although the routes of application are the same the present study has resulted in a higher tumour incidence—in spite of the fact that *Bianchi-Sforzi et al* (1) fed 32 times as much DMBA (8 mg) as was fed in the present study (0.25 mg). And finally no difference in the tumour incidence was found in the present group II and III (same strains) though the DMBA was applied orally and intraperitoneally respectively.

A third factor influencing the incidence and time of tumour develop-

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Marchant (11) painted the skin of mice aged 2 $\frac{1}{2}$ to 4 months 6 times at fortnightly intervals, the total dose of DMBA being 0 mg. 7 months after first skin painting she found 70 per cent of the mice with predominantly unilateral ovarian tumours.

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A third factor influencing the incidence and time of tumour develop

ment might therefore be found in the age at which the animals are treated with DMBA. In the present experiments the carcinogen was applied to immature mice (at the age of 21 days) while the animals used in *Leferre's* (9) *Marchand's* (11) and *Biancifiore et al's* (1) experiments were mature at the time DMBA treatment was begun (11 to 1 months of age).

Kaplan (8) found a higher incidence of X-ray induced ovarian tumours when the mice were irradiated at the age of 1 month (im maturity) than when they were irradiated at later ages (in maturity). Within the immature period of life *Peters & Levy* (12) found a maximum radiation sensitivity of mouse ovaries at the age of 21 days.

The age of 21 days was therefore chosen for DMBA treatment hoping to obtain a high incidence of ovarian tumours and it was presumed that the damage to the ovaries of the two tumorigenic procedures i.e. X irradiation (3, 10) and DMBA treatment might be identical.

Pathology and Oocytes

The pathologic changes in the DMBA treated ovaries which occur before tumour growth is established (the pre tumorous changes) are: Degeneration of follicles appearance of empty rings and pseudo follicles increase and luteinization of the stroma and persistence and merging of corpora lutea. These changes progress as the number of oocytes regress. From which cellular component(s) the tumour ultimately develops in the DMBA damaged ovary cannot be discussed on basis of the present material.

The fate of the oocytes during ovarian tumorigenesis is only partly known. It has been reported that the follicles (which contain the large oocytes) gradually disappear from the ovaries before tumour growth takes place whether the stimulus was DMBA treatment (1, 11) X-ray irradiation (3) or transplantation of an ovary to the spleen of a gonadectomized host (2).

In a study on X-ray induced ovarian tumours in mice (2) it was noted that the primary follicles had disappeared from the ovaries 7 days after irradiation of mice aged 3 days. These ovaries developed tumours 9 to 10 months after the irradiation. From the present experiments it appears that a destruction of the small oocytes is also the first recognizable cellular event that occurs in the ovaries after treatment with DMBA. It further appears that the pre tumour changes do not set in until the small oocytes have been considerably reduced in number. Tumour tissue and oocytes small or large were not found together in the same ovary. Consequently it is likely that a destruction of all oocytes is a condition sine qua non for neoplastic growth in the ovaries. However whether ovarian neoplasia inevitably will take place merely if the oocytes are destroyed is more uncertain. That this is probable is supported by the observation that all practically sterile

mice 12 mice born with only a few oocytes in their ovaries invariably develop spontaneous ovarian tumours (14)

The mechanism by which the DMBA or its metabolites acts upon the ovaries is still unknown. The author has shown that a single application of 0.25 mg of DMBA to 21 days old mice is sufficient to induce ovarian tumours in all treated mice after a latency period of 5 to 8 months. It is however not clear whether the carcinogen acts acutely for only a short period of time or whether its action continues until the tumour develops. Experiments have been started to determine how and for how long DMBA acts upon the ovaries following a single application.

The present study has already contributed to answer the first of these two questions. The first event in the ovaries following treatment with DMBA is a rapid destruction of the small oocytes. The small oocytes therefore seem to be the primary target cells of DMBA in the ovaries of immature mice.

SUMMARY

Immature female mice of two different strains were treated once with solutions of DMBA in olive oil by 3 different routes. Oocyte counts were done and histologic changes in the ovaries were described at different time intervals after treatment. The small oocytes were destroyed first followed by a reduction in the number of the growing and large oocytes. A characteristic sequence of pre tumourous pathologic changes in the ovaries was found which preceded neoplastic growth. Ovarian tumours occurred in all mice at 8 months following oral or intraperitoneal application of the DMBA and at 12 months after direct ovary painting with the carcinogen. An early development and a high incidence of ovarian tumours was found. The possible reasons for this are discussed in relation to 1) different sensitivity to DMBA of different strains of mice used, 2) different routes of DMBA application and 3) different sensitivity to DMBA in immature and mature mice. It is concluded as neoplastic growth in the ovaries occurs only after all oocytes have been destroyed that the small oocytes are the primary target cells of DMBA in immature mouse ovaries.

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By

POUL RANLOV & OLE WERDELIN

Received 19 vi 66

In a recent report we were able to demonstrate the regular development of spleen amyloidosis in normal C3H mice 2 to 6 days following the intravenous administration of 10^4 spleen cells from syngeneic donors previously hyperimmunized with casein (Werdelin & Ranlov 1966). After the cell transfer the recipient mice were treated with nitrogen mustard serial killings 1 2 3 4 and 5 days after the spleen cell transfer showed no amyloid formation in the recipient spleens until after 48 hours when some of the recipients developed thin incomplete rings in the perifollicular regions of the Malpighian corpuscles. During the following days of nitrogen mustard treatment the incidence and severity of the amyloid lesions increased. At day 5 after the cell transfer all recipient spleens showed marked amyloidosis. In other organs amyloid could not be demonstrated. Control experiments employing 1) the transfer of spleen cells from normal non immunized syngeneic donors followed by nitrogen mustard treatment of the recipients 2) treatment with nitrogen mustard alone and 3) transfer of spleen cells from donors hyperimmunized with casein but omitting the nitrogen mustard treatment of the recipients all failed to produce amyloid lesions in the respective recipients. Therefore it was tentatively concluded that the production of amyloid in the recipient spleens depended on two consecutive events: 1) A prolonged casein stimulation of the donor prior to the cell transfer and 2) a suppression of the transferred cells with nitrogen mustard after the transfer thus illustrating the theory of a local cellular and biphasic pathogenesis of amyloidosis originally put forward by Teilmann (1964).

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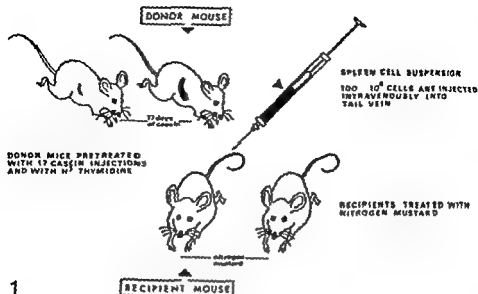


Fig. 1
General design of the experiment

recipients. In addition the amyloid deposits appeared more cellular than what is usually seen in experimental amyloidosis. Based on the assumption that the recipient amyloid was produced and precipitated *in situ* by the transfused spleen cells it was decided under similar conditions to transfuse isotopically labelled spleen cells from casein treated donors to normal syngeneic recipient mice subsequently treated with nitrogen mustard in order to define the route of migration of the donor cells and their possible relationship to the amyloid deposits eventually developing in the recipients.

MATERIAL AND METHODS

38 closely inbred C3H mice were used. They were aged from 2 to 4 months and the sex distribution was equal. They were at random divided into a donor group and a recipient group. In Fig. 1 the main principle of the experiment is outlined.

Donors. 12 mice were given a total of 17 subcutaneous injections administered daily as 0.5 ml of a 5 per cent solution of sodium caseinate 6 times a week. On the day after the last injection the donor mice were killed by cervical dislocation and the spleens were harvested and split 72, 48 and 24 hours before killing each donor mouse received intraperitoneally 0.5 microcuries of H³ thymidine per g body weight 3 hours before killing the same amount of H³ thymidine was administered intravenously. The H³ thymidine obtained from The Radiochemical Centre, Amersham (U.K.) had a specific activity of 5000 millicuries per millimole and was dissolved in normal saline so that 0.5 ml contained 10 microcuries of H³ thymidine.

Spleen cell suspensions. From each donor spleen a small piece was taken for microscopical counts. The rest was placed in a 15 Binger's tube and gently manipulated in a 1 cc syringe fitted with a 1/16 inch hypodermic needle. The cell suspension was washed 3 times in cold Binger's tube. It was adjusted so that 0.5 ml contained 10⁶ nucleated cells. As judged by the trypan blue test the viability ranged from 85 to 90 per cent.

Recipients 26 mice received each 10^6 nucleated spleen cells by intravenous injection in the tail 6 mice died within 15 minutes after the injection and these are in the following, referred to as group 0 The remaining 20 mice each received a subcutaneous injection of 0.05 mg nitrogen mustard (Frasol®) dissolved in 2 ml isotonic saline whereupon they were further divided as follows

- group I ■ mice killed 24 hours after cell transfer
- group II ■ mice killed 2 days after cell transfer
- group III ■ mice killed 3 days after cell transfer and
- group IV ■ mice killed 4 days after cell transfer

The recipients of the groups III and IV received an additional dose of 0.05 mg nitrogen mustard 48 hours after the cell transfer

The recipients were killed by ether inhalation Tissues were fixed in 10 per cent neutral formalin paraffin embedded and sections were cut 5 microns thick Amyloid was identified by its morphology in haematoxylin eosin and Papanicolaou stained sections and by its birefringence with Congo red under crossed polars

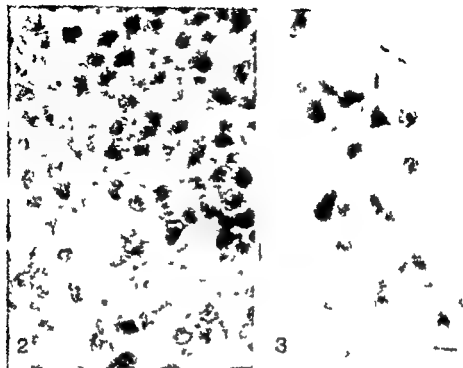
Autoradiography The deparaffinized sections were coated with Kodak Nuclear Research Emulsion (Ilford) stored at 4°C and developed after 70, 45 and 80 days of exposure After developing the sections were stained with haematoxylin eosin methyl green pyronin and alkaline Congo red A cell was considered labelled if 4 or more silver grains could be counted over its nucleus A parallel series of not coated sections was prepared for Papanicolaou staining

RESULTS

Apart from group 0 no spontaneous deaths occurred among the recipient mice

The distribution and incidence of amyloid deposits among donor mice and the various groups of recipient mice corresponded fairly well to those of our earlier reported similar experiments (Werdelin & Ranslow 1966) In the donor group small amounts of amyloid were found in the spleens of 6 out of 12 mice while no other organs showed amyloid lesions Among the recipients no animals of the groups 0 and I showed amyloid lesions while one out of 5 recipients in group II showed minute amounts of amyloid deposits in the spleen In group III 4 out of 5 recipients contained amyloid in the spleen while in group IV all 5 recipients showed significant amounts of splenic amyloid As a general rule the incidence and severity of the amyloidosis increased with the length of time after the cell transfer As was the case in our earlier reported experiments the amyloid was found exclusively in the perisplenic zones of the spleens of the recipient mice

Autoradiographic investigations Most labelled cells in the donor spleens (Fig. 2) were found in the perisplenic regions The majority of these were large cells with elongated chromatin weak nuclei containing one or two nucleoli A large proportion of these cells showed cytoplasmic pyroninophilia Other labelled cells were lymphocytes of various sizes and a few megakaryocytes Apart from the perisplenic zones the distribution of labelled cells throughout the splenic white and red pulps was rather uniform The labelling of the donor thymus was less pronounced most labelled cells being found in the thymic cortex In the donor liver relatively more Kupffer cells were found labelled than were the parenchymal cells



Figs 2, 3

- Fig 2 Aut radiograph of peritoneal region of donor spleen (white pulp at left right): labelled lymphoid and reticular cell many of them having extracellular pyroninophilic 45 days of exposure Methyl green pyronin ($\times 1400$)
- Fig 3 Aut radiograph of section of lung from recipient mouse killed 3 minutes after cell transfer showing accumulation of labelled donor cells in the capillary bed exposure time 10 days Haematoxylin eosin ($\times 1400$)

Figs 4

- Fig 4 Aut radiograph of spleen of recipient mouse killed 24 hours after transfer of hyperimmunized donor spleen cell labelled with ^{32}P thymidine in the central area of the Malpighian corpuscles (left) 3 days of exposure Alkaline (exposure $\times 1400$)
- Fig 5 Aut radiograph of recipient spleen 4 days after cell transfer in the white pulp 45 days of exposure Methyl green pyronin (1400)
- Fig 6 Aut radiograph of spleen from recipient mouse killed 10 days after cell transfer labelled cells are seen in the peritoneal region of white pulp in addition to the other grains have appeared in the peritoneal reticular and thymic cells 45 days of exposure (1400)
- Fig 7 Aut radiograph of recipient spleen 4 days after cell transfer are seen in the peritoneal region and in the red pulp (left) new formed small lymphoid cells have appeared in the spleen (1400)

In the animals of the *recipient group II* labelled cells were found exclusively in the capillaries of the lungs (Fig. 3). No labelled cells could be recognized in the recipient spleens or livers. The labelled cells were of the same type as those seen in the donor spleens many of them being pyroninophilic. Quite often they could be seen in clusters forming small emboli. In the recipient *group I* 24 hours after cell transfer practically all labelled cells had disappeared from the lungs and reappeared within the Malpighian corpuscles of the spleens. In each section usually 8-20 labelled cells could be found distributed over a single Malpighian corpuscle with a tendency to accumulate in the more central parts near the central vessel (Fig. 4). The labelling was confined to the nuclei. At this stage only an occasional labelled cell could be recognized in the perifollicular region and labelled cells were never found in the splenic red pulp 2 days after cell transfer in the recipient *group II*, labelled cells were still confined to the splenic white pulp but now a tendency to accumulate in the perifollicular regions was apparent (Fig. 5). The majority of labelled cells were of the large pale variety many being pyroninophilic. At this stage labelling in the splenic red pulp was seldomly seen. In the lungs the liver, thymus, intestines and kidneys extremely few labelled cells could be recognized. In the recipient *group III*, killed 3 days after the spleen cell transfer the labelled cells seemed to have migrated further out now being found in the splenic red pulp and in the perifollicular zone of the admittedly now somewhat disorganized and shrunken Malpighian corpuscle (Fig. 6). In 4 out of 5 animals of this group this localization coincided with the appearance in the same areas of small new formed amyloid deposits. These findings were repeated in the recipient *group IV* killed 4 days after transfer. In these animals significant amyloidosis was found in all the spleens. The labelled cells were found in the same localizations as those of group III. As was the case in the other recipient groups practically no labelling was found over other tissue sections neither was amyloid (Fig. 7).

In addition to the well-defined nuclear labelling hitherto observed now isolated silver grains appeared over the perifollicular regions of the recipient spleens. Most of these grains were apparently related to the cytoplasm of reticulo-endothelial cells probably representing phagocytized nuclear material derived from the labelled donor cells.

DISCUSSION

The main observations of the experiment presented above are: firstly the major part of spleen cells from mice hyperimmunized with casein apparently migrate to the perifollicular regions of the spleen when injected intravenously into normal syngeneic recipients subsequently treated with nitrogen mustard. This assumed migration is completed in the course of 2-3 days and seems to take place via the central vessel

of the Malpighian corpuscle. Secondly the appearance of labelled cells in the perifollicular zone of the recipient spleen coincides with the appearance of amyloid deposits in the same region. Finally the nitrogen mustard treatment necessary for the amyloid formation in the recipients seems to be accompanied by a significant destruction of the donor cells present in the recipient spleens.

In the literature dealing with the fate and the organ distribution of transfused lymphoid cells conflicting opinions have been expressed. These are mainly due to variations in genetical donor host relations, variations in the type of donor cells employed and most often variations in the intervals between the transfusion and the killing of the recipient animals. In general though most investigators agree that a major part of such transplanted cells after a certain length of time may be traced to the recipient spleens and to a lesser extent to the livers.

In the present experiment animals dying within 15 minutes after the transfusion showed practically all of the injected labelled cells concentrated in the capillaries of the lungs. This is in accordance with the findings of Osogoe (1950) and Keohane & Metcalf (1958) who found injected lymphoid cells accumulating in the capillaries of the lungs immediately after the injection after which they were gradually released into the blood stream. In the present experiment 1 and 2 days after cell transfer labelled cells were found predominantly in the splenic white pulp after 24 hours with a tendency to accumulate around the central vessel. This is in agreement with the observations of Gowers (1962). He found that between 3 and 12 hours after the transfusion of labelled lymphoid cells they could be found accumulated immediately around the central vessel in the white pulp of the recipient spleen. This was true regardless of the genetic relation between donor and host. In guinea pigs followed for 24 hours after the transfer of (allogeneic) isotopically labelled (Cr^{51}) lymphoid cells Turk (1962) found about one half of the activity remaining in the liver throughout the period while the activity in the spleen increased steadily from 1 to 6 per cent. With a similar technique Najarian & Feldman (1965) studied the rate of isotope disappearance from the spleens of mice injected intravenously with H^3 labelled spleen cells from either syngeneic or allogeneic donor mice. In syngeneic recipients the calculated half life of labelled cells was 82 hours in contrast to 40 hours in allogeneic recipients. In an allogeneic donor host system Haller *et al* (1964) found the majority of injected labelled spleen cells accumulating for 2-3 days in the recipient spleen. In a rather indirect way the fate of injected allogeneic spleen cells was determined by Haller (1964). He found the mortality from "runt" cut in half by removing the spleen of newborn mice shortly after the injection of allogeneic adult spleen cells. His observations suggest that the injected spleen cells immediately settle out preferentially in the spleen of the new host from

which they initiate the lethal graft versus host reaction. From 4 to 21 days after the transfusion of allogeneic adult lymphoid cells to neonatal rats Vowell & Defendi (1964) found the injected cells proliferating more actively in the lymph nodes than in the spleen. In accordance with others (Davies & Doak 1960; Howard, Michie & Simonsen 1961; Fox 1962) their findings suggested the splenomegaly occurring later during the graft versus host reaction being mainly due to host cell proliferation. These observations suggest that the sojourn of injected lymphoid cells in the recipient spleen may be a temporary one perhaps limited to a few days. However, that the strain combination may be decisive for the fate of injected parental cells must be emphasized. Fox & Howard (1963) found dividing cells of donor origin dominating the spleen after 3 weeks of graft versus host reaction in the strain combination C57Bl \rightarrow (C57Bl \times (BA)1; From these observations it appears that conclusions regarding the fate of injected lymphoid cells into syngeneic recipients should not be drawn without criticism from experiments employing allogeneic or xenogeneic strain combinations.

Murray & Murray (1964) found H³ labelled thymus cells injected intravenously into syngeneic mice after a few hours sojourn in the capillaries of the lungs migrating primarily to the spleen and to some extent to the bone marrow and the lamina propria of the small intestine. In many instances these authors found evidence of transformation into other cell types (blast like) and they refused to rule out the possibility of a direct incorporation of labelled material into phagocytic cells.

That the origin of the "lymphoid" cells transferred to syngeneic recipients may be decisive for their destination within the recipient was illustrated in an interesting report by Parrot *et al.* (1966). These authors describe the preferential localization immediately around the central arteriole of the Malpighian corpuscle (the "thymus dependent area") of injected labelled thymus cells. Similarly labelled spleen cells also accumulate in these areas but in addition are distributed at the periphery of the splenic follicles. Many more spleen than thymus cells enter the lymphoid tissues and the spleen appears to be the primary target. Earlier experiments by Diderholm & Lichtelius (1951) with guinea pigs showed that transfused P³² labelled thymocytes and lymph node lymphocytes both could be traced to the red pulp of the recipient spleens mainly perifollicularly though thymus cells commonly could be recovered in the spleen to a greater extent.

Migration to the thymus of transfused lymphoid cells seems to be an uncommon finding. Galton & Reed (1966) injected H³ labelled spleen cells from syngeneic donor mice into normal recipients. After 3 days the vast majority of labelled cells was recognized within the Malpighian corpuscles of the recipient spleens and in addition a few labelled cells appeared in the thymus glands.

Even in syngeneic combinations difficulties may arise. Recently *Celada* (1966) showed that mouse spleen cells in the donor animal primed to form antibody against human serum albumin failed to show the secondary response when challenged after transfer into intact mice of the same genotype. This barrier which severely affected the donor cells' capacity to implant and/or to function in the syngeneic system was found to be radiosensitive and to depend on the age of the recipient (being maximal after 2 months of age). In an allogeneic system *Hattler et al.* (1964) found that donor cells presensitized with an indifferent antigen had survival times similar to normal donor cells.

In the present material the relationship in time and topography between the appearance of labelled cells and amyloid deposits in the recipient spleens seems suggestive of a direct or indirect participation of these cells in the local production of the amyloid substance. Evidence that amyloid is formed *in situ* by cells belonging to the reticuloendothelial system was first presented by *Teitum* (1956, 1964). The present and our earlier experiments allow no conclusions to be drawn regarding the type of cells involved in this presumed local production of amyloid. The majority of labelled cells found in our recipient spleens were apparently of the reticular variety but may not necessarily be identical with the large pale cells observed in the donor spleens, as transformation into lymphoid type cells or *vice versa* under these conditions is known to occur (*Diderholm* 1961). Further, less than 20 per cent of the nucleated donor cells were labelled under the conditions of the thymidine treatment and moreover the composition of this labelled cell population did not reflect that of the whole transfused spleen cell population.

SUMMARY

Isotopically (H^3) labelled spleen cells derived from donors hyperimmunized with casein were transferred intravenously to normal syngeneic mice subsequently treated with nitrogen mustard. The recipients were killed in series 0, 1, 2, 3 and 4 days after the cell transfer. From 2 to 4 days after transfer all of the recipient mice developed splenic amyloidosis. Autoradiographic investigations revealed that the major part of the hyperimmunized donor spleen cells apparently migrated to the perifollicular regions of the recipient spleen. This assumed migration was completed within 2-3 days and seemed to take place via the central vessel of the Malpighian corpuscle. The appearance of labelled cells in the perifollicular zone of the recipient spleen coincided with the appearance in the same region of new formed amyloid deposits.

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SUBCUTANEOUS GROWTH OF EHRlich'S ASCITES CARCINOMA AFTER SUBLETHAL WHOLE BODY IRRADIATION

By

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Received 17 XI 66

It has been shown previously that the early intraperitoneal growth of the Ehrlich ascites carcinoma (LAC) is not changed after prior treatment of the host with whole body irradiation in sublethal doses (Thunold 1966a b). As this treatment was shown to abrogate the immune reactions the experiments indicate that this response is not of decisive importance to the intraperitoneal growth of LAC under these conditions. Recent work however has shown that an increase in spleen weight and histological changes in the spleen and lymph nodes characteristic of antigenic stimulation occur during both intraperitoneal and subcutaneous growth of LAC (Thunold 1967). Contrary to intraperitoneal growth subcutaneous tumour growth seems to be influenced by these host reactions.

The present work was planned to see if irradiation of the host 24 hours before transplantation might have any effect on the subcutaneous growth of FAC. Changes in spleen weight and histology were examined 24 hours and 8 days after irradiation in non tumour bearing mice and 8 days after irradiation in tumour bearing mice.

MATERIALS AND METHODS

Mice These were adult males and females of the closed colony kept at this Institute. The mice were of similar age the males weighing between 20 and 22 g and the females weighing between 21 g and 23 g. Two groups consisting of 12 females and 3 control groups of 5 males and 8 females were set up.

Tumour The Ehrlich ascites carcinoma used was a 12 day old ascites from a female mouse of the closed colony.

Irradiation Whole body irradiation (300 R) was given 24 hours before experiment (Thunold 1966a).

Experimental procedure

Tumour growth and histology Whole tumour ascites (25 / 160 cell in 0.1 ml ascites) was grafted subcutaneously on the back to 4 groups of 3 male and 12 female mice which had been irradiated or sham irradiated 24 hours previously. The mice were killed 8 days after inoculation on the 7th post transplantation day. After removal

TABLE 1

The Mean Tumour Weight after Subcutaneous Injection of Ehrlich's Ascites Carcinoma in Control (EAC) and Previously Irradiated (EAC + IRR) Mice. The absolute and relative spleen weights are given in mice receiving irradiation only 24 hours and 8 days previously and in tumour bearing mice. Entries means \pm S.E.

Treatment	No and sex of animals	Tumour wt (mg)	Absolute spleen wt (mg)	Relative spleen wt
NIL	8 ♂	—	87 \pm 12	0.39 \pm 0.05
	8 ♀		82 \pm 90	0.35 \pm 0.03
IRR (24 hours)	8 ♂	—	40 \pm 6	0.15 \pm 0.02
	8 ♀		41 \pm 4	0.18 \pm 0.01
IRR (8 days)	8 ♂	—	66 \pm 8	0.26 \pm 0.03
	8 ♀		52 \pm 17	0.23 \pm 0.07
EAC	12 ♂	143 \pm 45	250 \pm 73	0.93 \pm 0.31
	12 ♀	61 \pm 49	302 \pm 97	1.24 \pm 0.35
EAC + IRR	12 ♂	155 \pm 57	209 \pm 26	0.76 \pm 0.12
	12 ♀	149 \pm 36	183 \pm 17	0.77 \pm 0.09

of the tumours through a dorsal skin incision both tumours and animals were weighed. Sections from formalin fixed tumours and from the tumour bed were stained with haematoxylin and eosin.

Spleen weight and histology. The spleen was removed in tumour bearing animals and the spleen weight recorded to the nearest 0.001 g. The relative spleen weight was calculated using the formula $100 \times \text{Wt of spleen (g)}/\text{Wt of animal (g)}$. The relative spleen weight was also calculated in one untreated control group and in two

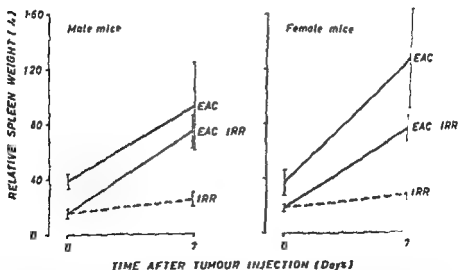


Fig. 1

The increase in relative spleen weight during growth of Ehrlich's ascites carcinoma (EAC) in normal and previously irradiated (IRR) male and female mice. The broken lines represent the change in relative spleen weight in mice without tumours between 24 hours and 8 days after irradiation.

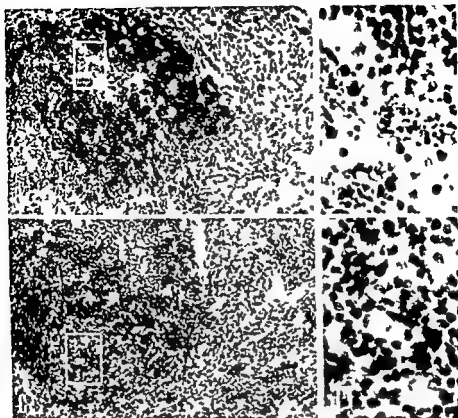


Fig 2

- a Spleen from non tumour bearing mouse 24 hours after irradiation. Oedema and large amounts of cellular debris and polymorphonuclear cells can be seen in the Malpighian body (left). There is no obvious changes in the red pulp. Haematoxylin and eosin $\times 150$.
- b Greater magnification ($\times 610$) of the cellular debris seen in the inset in a.
- c Spleen from non tumour bearing mouse 8 days after irradiation. There is less cellular debris in the Malpighian body (left) and that remaining is more closely packed. Haematoxylin and eosin $\times 150$.
- d Greater magnification ($\times 610$) of the cellular debris seen in the inset in c. This finding indicates phagocytosis.

Groups of mice which had received irradiation only 24 hrs and 8 days were only respectively. This was done as a control of the radiation effect. Sections were stained with haematoxylin and eosin and methyl green on a grid and examined microscopically.

RESULTS

Irradiated Non-tumour Bearing Mice

The changes in spleen weight after irradiation are shown in Table 1 and visualized in Figure 1. A significant decrease in relative spleen weight was found 24 hrs and 8 days after irradiation in both males (24 hrs



Fig 3

- a Spleen from non irradiated tumour bearing mice showing hyperplasia of the Malphigian body with activated germinal centre (left) The red pulp (right) is cellular and contains a number of erythrocytes (Haematoxylin and Eosin $\times 150$)
- b Spleen from non irradiated tumour bearing mice with relative absence of pyroninophilia in the Malphigian body (left) The red pulp contains a great number of pyroninophilic cells and some megakaryocytes (right) Methyl green pyronin $\times 150$

$P < 0.001$ 8 days $P < 0.05$) and females (21 hrs $P < 0.001$ 8 days $P < 0.01$) While the relative spleen weight showed no sex difference in control mice or in mice irradiated 9 days previously it was greater in female than in male mice 24 hours after irradiation ($P < 0.01$) No changes occurred in body weight after irradiation

Histological examination of the spleens irradiated 24 hours previously revealed cellular debris and polymorphonuclear cells scattered in the Malphigian bodies (Figs 2 a b) At 8 days after irradiation there was less cellular debris and that remaining was more closely packed (Figs 2 c d) No obvious changes could be seen in the red pulp

Non Irradiated Mice Receiving IAT

The mean tumour weight (Table 1) 7 days after transplantation was significantly greater in male than female mice ($P < 0.001$) Histologically the tumours showed small central areas of necrosis and haemorrhage The connective tissue in the tumour bed showed only infiltration of tumour cells and inflammatory cells but no organized tumour tissue was seen



Fig. 3

- a Spleen from irradiated tumour bearing mouse 8 days after irradiation. Resting Malpighian body with some foci of cellular debris (left) and a less cellular red pulp. Haematoxylin and eosin $\times 150$
- b Spleen from irradiated tumour bearing mouse 8 days after irradiation. The Malpighian body (left) is relatively free from pyroninophilia and only a few pyroninophilic cells can be seen in the red pulp. Methyl green pyronin $\times 150$
- c Greater magnification ($\times 610$) of pyroninophilic cells seen in the red pulp in b

The increase in relative spleen weight after tumour growth was significant in both sexes ($P < 0.001$). The increase was more pronounced in female than male mice (Table 1 and Fig. 1). Histological examination of the spleens revealed increased mitotic activity in the germinal centres and a cellular red pulp with erythropoietic activity, many pyroninophilic cells and a number of erythrocytes (Figs 3 a, b). This is in line with the previous experiment (Thunold 1967).

Irradiated Mice Receiving EAC

The mean tumour weight was similar in male and female mice in both groups (Table 1). Compared to the control female mice, the spleen weight in irradiated female mice was increased ($P < 0.001$). No significant difference appeared between the male groups. Histological examination of the spleens of these mice showed a cellular red pulp similar to those seen in the spleens of the control and the irradiated tumour bearing mice. It was concluded to be the same.

The increase in relative spleen weight during the tumour growth in irradiated mice was significant in both sexes ($P < 0.001$). This increase

was the same in the two sexes and it was remarkably similar in all animals as judged from the standard deviation of the means which were only $\frac{1}{2}$ to $\frac{1}{4}$ of the standard deviation in tumour bearing controls (Table 1 and Fig. 1)

The Malpighian bodies in these mice showed no mitotic activity and were less prominent than in the non irradiated tumour bearing controls. The red pulp was also distinctly less cellular than in the controls (Fig. 4 a) but some large pyroninophilic cells could be seen (figs 4 b c)

Microscopy did not reveal any sex differences

DISCUSSION

In this experiment the same growth pattern of subcutaneous transplanted I AC was observed in non irradiated mice as demonstrated previously (Thunold 1967) as the male mice showed large tumours than the females. In contrast there was no sex difference in tumour growth in the irradiated animals. While the tumours in the two male groups were of equal size tumour growth in irradiated female mice was increased compared to the controls. There was slight and similar tumour cell infiltration of the surrounding tissue in all groups but no organized tumour tissue was seen in the tumour bed. This showed that tumour dissection could be considered satisfactory.

These group differences in tumour growth may be related to the differences in lymphoid reactions. In the non irradiated mice the females showed a higher degree of splenomegaly than the males while the splenic enlargement was similar in the two sexes in the irradiated group. The splenic enlargement in the non irradiated mice was accompanied by histological evidence of antigenic stimulation while the spleens of the irradiated tumour bearing mice showed less accumulation of large pyroninophilic cells and plasma cells in the red pulp and smaller Malpighian bodies with no distinct formation of germinal centers. The status of pyroninophilic cells as producers of antibody has been well established (Lagrenus 1948, Coons *et al* 1955) and evidence is accumulating for involvement of the germinal centers in the same process (Gullin *et al* 1955, Ortega & Mellors 1957, Mellors & Kornfeld 1963). The histological findings thus indicate less immunological activity in the irradiated mice.

As depression of the immunological activity by sublethal irradiation measured by histological criteria is accompanied by increased tumour growth in female mice the present experiment supports the previous suggestion (Thunold 1967) that the immune response of the host is of consequence to the subcutaneous growth of I AC in this sex.

Decrease in immunological function of the mouse spleen after total body irradiation (400 R) has been studied by Makinodan (1962) who found an approximately 98 per cent decrease in agglutinin production

Using similar doses of X ray i.e. sublethal doses as in the present experiment *Argyris* (1963) found a similar decrease in immunological activity as measured by the ability of spleen cells to induce a graft versus host reaction. The quoted data and the increased tumour growth in irradiated female mice in the present experiment may be attributed to cellular damage to the reticuloendothelial system caused by the previous irradiation. A severe initial destruction of the germinal centres and loss of spleen weight was found 24 hours after irradiation in the present experiment i.e. at the time of tumour injection with substantial regeneration 8 days after exposure. These findings are comparable with those published by others (*Henshaw* 1941; *Brecher et al.* 1948; *Murray* 1948) on the effects of similar doses of X irradiation on the spleen and lymph nodes of mice.

The previous (*Thunold* 1967) and the present experiment show histological changes in lymph nodes and spleen indicative of a host immune response during growth of EAC. This homotransplantable tumour thus seems to contain antigenic components which are not shared by the host. The acceptance and progressive growth of the tumour in spite of this antigenic difference appears to be dependant on two main factors: the size of the inoculum and the immunological defence of the host. The dose used in these experiments is of a magnitude which seems to overwhelm the defence mechanism in the male sex while tumour growth in female mice is effected by a greater immune response in this sex.

SUMMARY

Subcutaneous growth of the Ehrlich ascites carcinoma has been investigated in normal and previously irradiated mice and related to changes in spleen weight and histology.

In non irradiated mice tumour growth was greater in the males than in the females. The female mice showed a greater increase in spleen weight than the males and the spleens of both sexes showed histological evidence of immune response. This suggests that tumour growth in the females has been inhibited by their greater immune response to the EAC.

This is supported by the fact that when the immune defence mechanism of the mice is depressed by whole body irradiation, as measured by histological criteria, no sex difference is apparent in tumour size or host reaction and tumour growth is significantly greater than in non irradiated females.

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PRIMARY GERM CELL TUMOR OF THE
ANTERIOR MEDIASTINUM
WITH FEATURES OF ENDODERMAL
SINUS TUMOR
(*Mesoblastoma Vitellinum*)

By

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Received 16 VII 66

The anterior mediastinum and the pineal gland share the curious property of being the site of predilection for the development of the extragonadal germ cell tumors. Teratomas, embryonal carcinomas, chorioepitheliomas and seminomas have been described in these locations (Friedman (5) Kountz *et al* (7) Oberman *et al* (10) O'Gara *et al* (11) Inada & Nakano (8) Lattes (9)). In contrast to the adult testes however most of the germ cell tumors in extragonadal location are benign. The reason for this difference and for the location of primordial germ cells in these organs is unknown. The study of this interesting group of neoplasms is impaired by their rarity and justifies the reporting of single cases. In the ovary and in the testis Teilum (15, 16) described germ cell neoplasms characterized by a mixture of undifferentiated neoplastic mesoblastic cells and irregular spaces closely resembling the endodermal sinuses of Duval characteristic of the rat placenta (Duval (+)). The author considered this type of neoplasm as an extraembryonic membrane tumor morphologically distinct from the extremely rare group of the embryonal carcinomas. The following report of a case of anterior mediastinal primitive germ cell tumor with endodermal features is the first published documentation of such a tumor in the anterior mediastinum. The case is of extragonadal origin. We believe that in more cases will be recognized and the characteristic features of this neoplasm will become more well known.

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CASE REPORT

A thirty three year old Negro man was admitted to Michael Reese Hospital and Medical Center because of fatigue weight loss mild substernal pain and cough productive of black stained sputum. His symptoms had initiated ten months earlier and had become progressively more severe during the past four months. The family and personal history was non-contributory. On admission physical examination disclosed no significant findings other than moderately enlarged left cervical and supraclavicular lymph nodes. Laboratory data were within normal range. A chest x ray and tomogram revealed a large anterior mediastinal mass which displaced the trachea posterior. The clinical impression was that of a malignant lymphoma or a thymoma although the possibility of a teratoma could not be entirely ruled out. Biopsy of the supraclavicular lymph nodes revealed only non specific lymphoid hyperplasia.

On the seventh hospital day mediastinoscopy revealed a large tumor mass which occupied the anterior mediastinum surrounded the large vessels and extended into the right pleural space. The mass was of deep gray purple color and was extensively necrotic. It could be only partially resected in view of its relationships with the superior vena cava and the innominate vein.

Following surgery the patient made an uneventful recovery although cytological examination of the sputum revealed the presence of malignant cells indicating extension of the tumor to the tracheo bronchial wall. He was discharged two weeks later and followed in the out patient department. He received 12,210 r to the anterior mediastinum in twenty seven treatments over a period of thirty eight days. He appeared to tolerate the treatment well however two months following his first admission he noticed bilateral subcutaneous masses in the anterior chest wall which were considered as metastases. For these additional 5000 r were administered by means of Co 60 radiation. In spite of therapy he became progressively weaker had repeated hemoptyses and became markedly anemic. The second admission was four months following discovery of the tumor. At this time there were left supraclavicular and posterior cervical lymphadenopathy and left pleural effusion. A test for urinary gonadotropin was negative. The patient received Cytosan Nitrogen mustard and Tetracycline intrapleurally. Despite all therapeutic efforts he declined rapidly and expired in marked respiratory distress five months after the initial diagnosis had been made.

Pathology

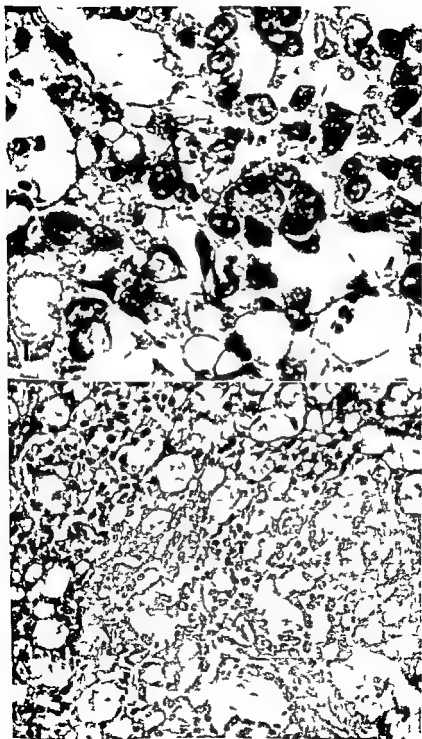
The surgically removed tumor consisted of a partially encapsulated gray purple mass which measured $11 \times 8 \times 6$ cm and weighed 209 gm. On section it was gray purple in color soft centrally necrotic and hemorrhagic.

Microscopically the tumor was composed of several cell types. The main portion was formed by solid masses of large cells with clear cytoplasm and round to pleomorphic nuclei with delicate chromatin and prominent nucleoli (Fig. 1). Intermingled were areas of stellate cells connected by delicate projections and forming a loose network with wide meshes (Fig. 2). Imperceptibly merging with these areas of undifferentiated cells were irregular spaces lined by low cuboidal or

Fig. 1 *

Fig. 1 The tumor is composed of solid nests of large undifferentiated cells with clear cytoplasm. Some of the cells have large cytoplasmic vacuoles. Note the cystic spaces lined by flattened cells. (H.E. $\times 613$)

Fig. 2 Areas showing clumps of markedly vacuolated cells forming delicate web-like structures with developed microvilli. (H.E. $\times 613$)



flattened cells with large round or indented hyperchromatic nuclei and scanty cytoplasm. The cavities appeared to communicate with each other forming an intricate system of irregular channels in which papillary structures projected (Fig. 3). The papillae were covered by a single layer of epithelial like cells supported by delicate connective tissue containing thin walled blood vessels. On cross section these papillary structures appeared as peculiar glomerularlike formations with a single capillary in the center covered by a mantle of cylindric cells of epithelial character (Fig. 4). These structures (Fig. 5) have been interpreted as invaginated vitelline endoderm accompanying the vessels of the extra embryonic mesenchyme (15-16). In the cytoplasm of both the flattened cells and in the epithelial like cells there were eosinophilic strongly PAS positive droplets. In addition extracellular accumulations of such hyaline globules were often present (Fig. 5).

At the periphery the tumor mass was partially surrounded by dense collagen in which residual thymic tissue was present (Fig. 6). Within the tumor mass were small lymphoid foci possibly representing residual lymphatic tissue of the thymus.

As the cells of the sputum appeared quite unusual a description of these may be of interest. The cells appeared mostly single or in pairs. Some of the cells had large cytoplasmic vacuoles, large nuclei which appeared round or oval with distinct nuclear membranes and finely dispersed chromatin. Some were hyperchromatic, lobulated and irregular in outline with chromatin clumping (Fig. 7). The nuclei varied in size and shape.

At autopsy the original surgical site was entirely obliterated by tumor regrowth and the anterior mediastinum beneath the sternum was occupied by a large yellowish gray tumor mass with multiple areas of hemorrhagic necrosis and an outer layer of intense reddish brown soft tumor tissue extending to the entire left parietal and visceral pleura and lung. The thymus gland was not recognized as such. Hemorrhagic and sero-fibrinous fluid filled most of the left pleural space with subsequent compression of the lung. On the right side the pleural lining and cavities were free of tumor however there was presence of confluent bronchopneumonia and multiple thromboemboli within the peripheral vessels of the lungs.

In addition to local extension the tumor had metastasized widely throughout the body. Almost all of the thoracic lymph nodes were involved bilaterally including the mediastinal hilar and peribronchial lymph nodes as well as the supraclavicular and paravertebral lymph

Figs 3-5

Fig. 3 Systems of irregular channels lined by low cuboidal epithelium with the papillary formations with a blood vessel in their centre. (a cross section of such structures vaguely resemble immature renal glomeruli. H.E. x 90)

Fig. 4 Endodermal sinus structure with perivascular mantling of cells. (H.E. x 210)





7a

7b

Fig 7

(a and b) Tumor cells in the sputum. Note in a the large oval shaped hyperchromatic nuclei and the scanty cytoplasm. In b the cell on the left has a large cytoplasmic vacuole. The remaining cells have round or lobulated nuclei clumping of the chromatin and nucleoli of varying size (Papanicolaou $\times 517$)

nodules. Metastases were present in the liver and in the skin of the left anterior chest wall. Careful attention was given to the examination of the testes. No tumor tissue was seen grossly or microscopically in any of the multiple sections examined by the method of serial blocks. Further, there were no areas of scarring in the testes. A striking feature was the widespread atrophy of the germinal epithelium and hyaline thickening of the tubular basement membrane. The tubules were lined by Sertoli cells and few scattered spermatozoa. The interstitial cells were well preserved and did not appear increased in number (Fig 8).

Microscopic examination of the tumor revealed essentially the same characteristic features seen in the surgical specimen (Fig 9). In addition many of the metastatic masses showed a more undifferentiated pattern. In some of the sections of the recurrent mediastinal tumor there were large cells with strongly eosinophilic cytoplasm and large hyperchromatic nuclei resembling syncytiotrophoblastic cells.

DISCUSSION

As illustrated in the Figs 1 through 5 and Fig 9 the anterior mediastinal neoplasm reflects the histologic features of the endodermal sinus tumor as described by Terlum (15-16) in the ovary and testis. These

Figs 4-6

Fig 4. Area of the mediastinal tumor showing the characteristic endodermal sinus structure representing yolk sac endoderm that expanded and dissected around the vessels of the extraembryonic mesenchyme. The structure is surrounded by a loose vacuolated network of stellate mesodermal cells and small cystic spaces showing focal accumulations of PAS positive hyaline globules (H.E. $\times 700$).

Fig 5. Well preserved thymic tissue at the periphery of the anterior mediastinal tumor. Note the Hassall's corpuscle (H.E. $\times 740$).



Fig. 8

Representative section of the testis. The tubules are lined by Sertoli cells and isolated spermatogonia. Note the marked thickening of the tubular basement membranes (H.D. $\times 60$).



Fig. 9

Pulmonary metastasis. The tumor although generally more uniform in cellular pattern, the metastases reveal typically the endodermal sinus pattern (H.D. $\times 150$).

are (a) areas formed by stellate mesodermal cells forming a loose vacuolated network delineating irregular spaces lined by flattened endothelial like cells and often containing foci of active hemopoiesis (b) endodermal sinus structures characterized by perivascular arrangement of cylindric epithelium which in cross section vaguely resemble immature glomeruli. They are surrounded by cystic spaces lined by a single layer of flattened mesothelial like cells with prominent nuclei. In longitudinal section they appear as a complete labyrinth resembling the endodermal sinuses of the rodent placenta (c) compact masses of undifferentiated cells similar to the undifferentiated cells of the developing embryo and (d) cystic structures lined either by flat mesothelial like cells or by low cuboidal epithelial like cells. These cysts are considered analogue to the yolk sac of the early embryo.

The proportion of the various patterns vary considerably not only in different tumors but also in different areas of the same tumor. In the present case the features mentioned in (d) were not seen. However there was a striking resemblance to the ovarian and testicular tumors described by *Teilm* (15-16) as endodermal sinus tumor with the testicular neoplasms of infant testis reported by *Huntington et al* (8) and with the sacrococcygeal teratoma of *Raghunatha et al* (12) also considered examples of endodermal sinus tumors.

In *Teilm*'s opinion this tumor represents the differentiation of entirely undifferentiated neoplastic embryonal cells (15-16) into extraembryonic membrane structures such as the mesoblast and yolk sac endoderm. *Teilm*'s interpretation of the histogenesis of these neoplasms was based on comparative studies which among other things showed a striking resemblance of the perivascular sinusoid structures in the tumor with the embryologically well defined endodermal sinus structures which are prominent in the rat placenta. In both instances these formations represent diverticula of yolk sac endoderm that expand and dissect around the vessels of the extraembryonic mesenchyme. While this comparison is valid not only in regard to the general appearance in cross and longitudinal sections but also to the type of lining cells it would not imply a specific capacity of the tumor to reproduce phases of placentation in the rodent (17).

The ovarian tumors show a far more uniform histologic pattern than their testicular counterparts in adults which usually reveal great variations of immature embryonic tissues. The term embryonal carcinoma has been a subject of criticism since it lacks clarity and leads often to confusion by its ambivalent use. For practical use the term may be retained to designate these complex germ cell tumors of the adult testis which comprise a fairly homogenous clinical group. However it is not sufficient basis for histologic characterization and recognition of the ovarian counterparts and similar tumors in the infant testis or extragonadal positions (16).

In our case the pattern of endodermal sinus tumor was striking in

the resected tumor but only found locally in the recurrent tumor and its metastases (Fig. 9). Clinically the neoplasm as the embryonal carcinoma of the adult testis affect the same age group and is resistant to radiation or chemotherapy. In contrast to the behaviour of this type of neoplasm in children all cases of endodermal sinus tumor in the adult have a malignant course regardless of treatment.

It is well known that gonadal malignant teratomas, embryonal carcinomas and choriocarcinomas may metastasize solely to the mediastinum. In this case however serial blocks of the testis failed to show any tumor or scar. In addition thymic tissue was identified at the periphery of the neoplastic mass (Fig. 6). It is then reasonable to assume that this tumor arose in the thymus gland or in its proximity. There is no agreement as to the factors which lead to the development of gonadal and extragonadal teratomas and embryonal carcinomas. Willis (18), Collins & Pugh (2) deny their origin from germ cells and believe that they arise from foci of plastic pluripotential embryonic tissue which have escaped from the influence of the primary organizer during fetal life. Most authors however believe in their origin from germ cells through different stages of development (Liedman (5)), Dixon & Moore (3), Teilum (15, 16), Bhargava & Reddy (1), Schlimberger's (13) interpretation that the mediastinal germ cell neoplasms arise from the third branchial pouch, the anlage of the thymus seems unlikely. It is now generally accepted that the germ cell neoplasms in this region are derived from germ cells which have been misplaced during ontogeny and that during their passage from the yolk sac endoderm they have travelled through the retroperitoneum to the mediastinum instead of the gonadal ridges (Teilum (17)). Of interest was the presence of a marked degree of atrophy of the germinal epithelium and the thickening of the basement membranes of the tubules of the testis. No explanation is readily available for this process. It may have been the result of invasion and chemotherapy although the latter appears unlikely in view of the findings of Oberman & Liecke (10). Three patients in their series of mediastinal malignant germ cell tumors had similar testicular findings. These authors do not state which one of the patients of their series had testicular tubular atrophy but only one was treated with nitrogen mustard, the others received only x-ray therapy or were surgically treated. No explanation has been offered to clarify the striking preference of the malignant germinal tumor for the male while the incidence of the benign counterpart is approximately the same in both sexes. Similar contrasting ratios of malignant and benign teratomatous tumors in the two sexes are seen also in the gonadal tumors. Here the suggestion has been presented ((7) p. 13 and (14) p. 226) that the environment in the female gonads predisposes germ cell neoplasms to differentiation whereas the male environment fosters undifferentiated growth. This hypothesis could be extended to extra-gonadal germ cell tumors. It is conceivable that in the female the same

genetic material has greater potency to differentiation possibly because of a more suitable endocrine environment. In this respect it is of interest that the endodermal sinus tumors—which in Teilmann's (15-16) view are extra embryonic and in fact endodermal derivatives from germ cells—are rare in the adult testis but common in the testis of the young child and in the ovary.

SUMMARY

The first case of mediastinal germ cell tumor with endodermal sinus features in a thirty-three year old Negro male is reported. The neoplasm exhibited the predominant pattern of communicating cavities lined by cuboidal or flattened cells and papillary structures as described in the ovary originally by Teilmann. The tumor was rapidly fatal and metastasized widely.

The specific histological features of endodermal sinus tumors in this and other regions will enable their proper identification and distinction from other types of neoplasm.

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THE RELATIVE BIOLOGICAL EFFECTIVENESS OF HIGH ENERGY RADIATION ON THE MITOTIC ACTIVITY IN NORMAL HEPATIC REGENERATIVE TISSUE

By

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Received 20 xii 66

The relative biological effectiveness (RBE) of some radiation r compared to some standard radiation s is defined by D_s/D_r where D_s and D_r are respectively the absorbed doses of the two beams in the region of interest that produce an identical biological response. As RBE can also be used the ratio of the radiation effects which can be produced by the same absorbed dose. In this work the latter definition is used.

The RBE of different types of radiation has usually been studied by indirect methods for example by total body irradiation of animals and observation of the 50 per cent mortality ($LD_{50/30d}$ = lethal dose_{50/30d} = the dose required to cause 50 per cent deaths of the irradiated animals within a 30 day period) as Paterson *et al* did. They irradiated mice with 300 kV roentgen rays and with γ rays from a 4 MeV linear accelerator. LD_{50} was achieved with 560 rad and the linear accelerator with 744 rad which gave an RBE of 0.743 ± 0.03 for the latter. Using irradiation of the thymus and the spleen and observing the weight loss the RBE was 0.858 ± 0.14 and the RBE for drosophila was 0.82.

Another method of RBE determination is based on tissue culture radiation. By irradiating for an observation period of 24 hours tissue cultured fibroblasts of chick heart and HeLa cells of pavement epithelium carcinoma of portio uteri.

Gartner (1955, 1958) among others determined the RBE for electrons (15 MeV) and compared the effect with that of 180 kV γ rays. He noted the number of mitoses, mitotic phases, injured mitoses and compared the effect of different types of radiation by using doses from 250 R to 2000 R at rates of 40 R/min and 1000 R/min. He found that the RBE values increased with the dose rate and total dose. The RBE with electrons was close to 1 when the dose rate was 1000 R/min and the total dose exceeded 500 R.

Gartner & Zoepfrits found that the number of mitoses was reduced

and that of pathological mitoses increased with an increasing total dose of beta radiation emitted by Sr^{90} . Bauer & Gärtner (1962) found that continued irradiation heightens the biological effects.

Total arrest of tumour growth after X-ray therapy and cobalt therapy was studied by Linden (1964) who determined the maximum dose that needed to destroy Walker cancer of the rat. X-ray radiation destroyed it with 2500 R and cobalt with 3500 R. The RBI value obtained from these results for the gamma radiation of cobalt was 0.72 ± 0.1 .

The relative biological effect has been studied comparatively little *in vivo* by quantitative methods in the high energy range. The present study is an attempt to explain this question *in vivo* by using different qualities of radiation and by giving one dose and comparing its effect on mitotic counts, phases and injured mitoses.

MATERIAL AND METHODS

Three month old white male rats (Sprague Dawley) were used for the experiment. Six groups each of 10 animals weighing 195-240 g were kept in the same cage at $+20^\circ\text{C}$. To produce regeneration of the liver 40 per cent hepatectomy was performed under ether anaesthesia by removing the median and left lobes according to the method of Higgins & Anderson (1931).

The time chosen for irradiations of varying energy was 49 hours after hepatectomy. This is when mitotic activity is at its highest after hepatectomy according to Lahti *harju* and it is thus possible to discern the effect of irradiation on the mitoses more accurately. The liver was then given a dose of 300 rad at a rate of 40 rad/min at maximum dosage as previously employed in some methods of irradiation by Lahti (1955, 1959). The 300 rad dose was chosen because it is the conventional single dose in radiotherapy. Radiation was given to the animals in the different groups by 200 kVp X-rays (HVL 1.6 mm Cu), cobalt ^{60}Co beta betatron X-rays and by 55 and 20 MeV betatron electrons. One group of 10 rats served as the control group. The irradiation time was at 7 p.m. and the technical methods were as follows.

The rat was surrounded by water equivalent material which consists of paraffin, beeswax and talc so that density is 1 g/cm³. Irradiation took place in a 8×8 sq cm field so that the rest of the liver was situated at the point of maximum dosage (Fig. 1). During irradiation the dose being delivered was monitored by an ionization

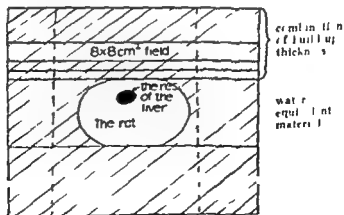


Fig. 1

The irradiation of the liver of the rat by different radiation.

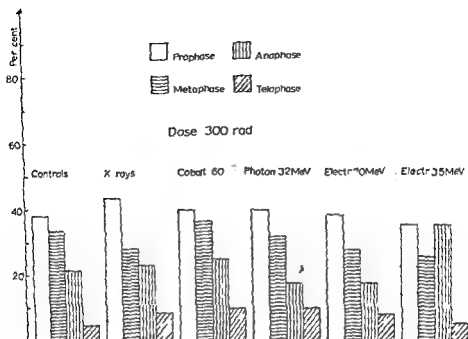


Fig 2

The mitotic phases after 12 hours by using different radiations

tion chamber which was checked against Max Planck Institute calibration according to Fritschko et al (1961). It can thus be assumed that the only differences involved in the irradiation were in the physical characteristics of the five beams and that it must be these therefore which are responsible for any biological differences observed. For the conversion of roentgens into rads the stopping power and density effect according to the National Bureau (1961, 1964) and Johns (1961) were taken into account.

The animals were decapitated 12 hours after irradiation. Tissue specimens were taken immediately from the liver, fixed in Bouin's solution and stained with hemalum-eosin. Mitoses were counted per 100 fields with a binocular microscope magnification $10 \times 100 \times 15$ over an area of 0.785 sq mm.

The following points were observed: mitotic counts, mitotic phases, pathological and multipolar mitoses.

RESULTS

The mitotic counts were highest in the controls (10 mitoses per 100 fields) (Table 1). X rays gave the lowest and 20 MeV electrons the highest mitotic index. After subtracting the mitotic indexes for different irradiation groups from the mitotic index for the control group, the RBE was calculated by dividing the remainder of each irradiation group by the remainder of the X ray mitotic index ($10 - 4.0 = 6.2$). The RBE values for the different irradiation groups are shown in Table 1.

There were no significant differences in the occurrence of different mitotic phases after exposure to different sources of radiation (Fig 2).

TABLE I
 Mitotic Index, Cytological and Multipolar Mitoses and RNF Value of the Liver after Hepatectomy
 72 Hours after Irradiation with γ rays (of all ^{60}Co Electrons and Helium Photons)

	C (micro)	γ rays	Each Group Comprises 10 Animals			
			Cobalt 60	32 MeV Electron	20 MeV Electron	35 MeV Electron
Mitotic Index Mean	10.2 ± 0.9	4.0 ± 0.4	4.6 ± 0.4	5.2 ± 0.9	7.0 ± 0.4	6.2 ± 0.9
Mitotic Index Mean		6.2 ± 1.2	5.6 ± 1.2	5.0 ± 1.7	3.2 ± 1.2	4.0 ± 1.8
RNF		1	0.90 ± 0.6	0.80 ± 0.31	0.52 ± 0.22	0.64 ± 0.31
Cytological Mitoses per cent	5.0	35.0	37.0	27.0	46.0	35.0
Multipolar Mitoses per cent	0.1	-	0.1	0.1	0.5	0.1
Mitotic count mean						

Prophases were most numerous in all the groups except in the 30 MeV electron group where prophase and anaphase phases were the same (35 per cent)

Pathological mitoses produced by irradiation were found in equal numbers after X rays cobalt and 35 MeV electrons some 30 per cent the maximum 46 per cent was noted after 20 MeV electrons and the minimum 27 per cent after photon irradiation Multipolar mitoses were also most frequent after 20 MeV electron irradiation (Table 1)

DISCUSSION

The time chosen for taking the biopsy specimen was 12 hours after irradiation since according to *Gärlnier et al* the rise in the mitotic index the secondary effect is at its highest 9–12 hours after irradiation of 250–2000 R in the tissue culture

X ray irradiation had the most pronounced effect on mitotic counts (4.0 ± 0.4 mitoses per 100 fields Table 1) and 20 MeV electrons the least pronounced (7.0 ± 0.4) The value for cobalt was 4.6 ± 0.4 When the RBE value of X rays was 1 that for cobalt was 0.9 ± 0.26 which exceeds the value reported by *Linden* (1964) in experiments with Walker cancer This suggests that the RBE in tumour is different from that in normal growing tissue The RBE values of photons were 0.80 ± 0.31 of 20 MeV electrons 0.52 ± 0.22 and of 30 MeV electrons 0.64 ± 0.31 The results for the different types of irradiation differ from one another very rarely Thus the RBE value cannot be regarded as reliable for different kinds of irradiation

Definite radiation damage was seen in dividing cells after the different types of radiation It was relatively most marked after 20 MeV electron radiation and the percentage of pathological mitoses was 46 Pathological mitoses were most numerous after electron radiation and fewest after photon radiation With all the sources of radiation pathological mitoses increased significantly compared with the controls They were most frequent in the metaphase and telophase—about 80 per cent—as *Friedrich et al* (1964) also noted when they irradiated the root tip cells of *Allium cepa* with 15 MeV electrons

SUMMARY

The regeneration of the liver of rats was produced by performing 70 per cent hepatectomy After two days the rest of the liver was irradiated with a dose of 300 rad at the rate of 40 rad/min Radiation was given by 200 kV X rays cobalt 32 MeV X rays of betatron photons and both 20 and 30 MeV betatron electrons Tissue specimens were taken 12 hours later from the liver and mitoses were counted per 100 fields X rays gave the lowest and 20 MeV betatron electrons the highest mitotic index The pathological mitoses were most frequent after electron

radiation lowest after cobalt irradiation. On the basis of the mitotic frequencies the RBI (relative biological effectiveness) of the different types of radiation was calculated taking the RBI for 200 kV X rays as 1. The RBI value for cobalt radiation was 0.9 ± 0.26 and for the fast electrons and photons the values were smaller.

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THE EFFECT OF HIGH ENERGY RADIATION ON THE MITOTIC ACTIVITY AND RELATIVE BIOLOGICAL EFFECTIVENESS IN WALKER TUMOURS

By

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The effect of different types of radiation has been studied by observing the $LD_{50/30d}$ (Lethal dose 50 per cent deaths in 30 days) Paterson *et al* (1957) in total body irradiation of mice and by irradiating tissue cultured normal cells or malignant cells and observing the mitotic activity and determining the relative biological effectiveness of electrons Gartner (1958) and Friedrich *et al* (1964)

The relative biological effectiveness (RBE) can be expressed either as a ratio of doses which produce a given effect or as a ratio of effects following equal doses. In this work the latter definition is used. In an early work by Voutilainen *et al* the same question was studied *in vivo* by irradiating the rest of the liver 2 days after 70 per cent hepatectomy when mitotic activity is at its highest. X rays gave the lowest and betatron electrons of 20 MeV the highest mitotic index. The RBE for X rays of 200 kV was 1 and for cobalt radiation 0.9 ± 0.26 . For the fast electrons and photons the values were smaller.

The tumour growth after X ray and cobalt therapy was studied by Linden (1964) in Walker cancer. The RBE value obtained for the gamma radiation of cobalt was 0.72 ± 0.1 .

The present study is an attempt to explain the effect of high energy radiation on the mitotic activity and RBE value in Walker cancer.

MATERIAL AND METHODS

Three month old white rats were used for the experiment. These CR to k rats with Walker cancer were supplied by the Chester Beatty Research Institute in London. Groups of 10 animals weighing 250-350 g were kept in the same cage at 20°C. The tumour was implanted under the skin of the interscapular region. After about 10 days when the tumour was spherical and about 1.5 cm in diameter the irradiation was given. The rat was surrounded by tissue equivalent material dimensions $70 \times 20 \times 20$ cu cm consisting of paraffin beeswax and talc and making up a density of 1 g/cu cm. The paraffin beeswax ratio was 2:1. The top of the tumour

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(a depth of 0.5 cm under the skin) was always situated at the point of measurement. The tumour absorbed dose was a constant 300 rads. Tissue specimens were taken after the irradiation in the region where the calculated thickness was 0.5 cm.

The radiation sources and the exposure conditions were as follows:

200 kVp X Rays. The standard beam was from a Siemens constant potential X-ray machine "Stabilipan 250" operated at 18 ma with a total filtration of 1.0 mm Cu HVL of 1.6 mm Cu. The distance from the X-ray target to the phantom surface was 50 cm giving a field size of 8×8 sq cm and the absorbed dose rate was 40 rad/min at a depth of 1 cm. The absorbed dose ratio of maximum to minimum was about 1.03.

^{60}Co γ Rays. A Siemens Cammatron - 1 teletherapy unit. The activity of the source was approximately 2000 curies. The distance from the source to the phantom surface was 91 cm giving a field size of 8×8 sq cm and a peak absorbed dose rate of 40 rad/min at a depth of 0.5 cm. The absorbed dose ratio of maximum to minimum was about 1.07.

32 MeV Photons. From a Brown Boveris Askleptron 35 betatron operated at 32 MeV. The distance from the target to the phantom surface was 100 cm, the field size was 8×8 sq cm and the peak absorbed dose rate approximately 40 rad/min at a depth of 5.5 cm. The absorbed dose ratio of maximum to minimum was about 1.

20 MeV Electrons. The electrons were accelerated by the same betatron as above. The distance to the phantom surface was 110 cm giving a field size of 8×8 sq cm and a peak absorbed dose rate of approximately 40 rad/min at a depth of 1.5 cm. The absorbed dose ratio of maximum to minimum was about 1.

35 MeV Electrons. As above.

Calibration for all five radiations was conducted with a 250 liter green chamber *Sinclair et al* (1958) and Baldwin Farmer sub standard dosimeter Mk. 2. Both were checked against Max Planck Institute calibration according to *Kretschik et al* (1961). The Fricke ferric sulphate dosimeter was used (*Schalek et al* 1962). The dose measurements and high energy electrons were compared at the betatron in Helsinki and Umeå, Sweden in December 1964 (*Hellinger* 1965). The dose values in Helsinki and Umeå were in good agreement with a maximum deviation of 3 per cent according to *Ellefsson et al* (1965). The comparison was performed with Fricke also against Vienna, Austria when the difference was less than 1 per cent (*Nagl et al* 1965). The conversion of röntgens into rads was calculated according to the National Bureau (1961 and 1964). The irradiation time was 7.3 m.

The animals were decapitated 12 hours after irradiation. Tissue specimens were taken from the tumour 0.5 cm under the skin, fixed in Bouin's solution and stained with hemalum-eosin. Mitoses were counted per 100 fields with a binocular microscope magnification $10 \times 100 \times 15$ over an area of 0.78 sq mm.

RESULTS

The mitotic counts of the controls and the different radiation groups are given in Table 1. They were highest in the control group (39.2 ± 2.7). In the irradiation material the X-ray group gave the lowest (9.9 ± 0.9) and the 35 MeV betatron electrons group the highest mitotic index (26.7 ± 2.9). The other mitotic counts were cobalt 13.1 \pm 2.1 photons, 21.4 \pm 1.9 and 20 MeV betatron electrons 21.1 \pm 2.0.

After subtracting the mitotic indexes for the different irradiation groups from the mitotic index for the control group the RBI value was calculated by dividing the remainder of each irradiation group by the remainder of the X-ray mitotic index ($39.2 - 9.9 = 29.3$). The RBI values for the different irradiation groups are shown in Table 1. The RBI values ranged from 1 to 0.38.

The pathological mitoses produced by irradiation were lowest in the cobalt group (13 per cent) and highest in the 32 MeV photon group (62 per cent) (Table 1).

TABLE 1
Mitotic Count Loss and RBF of Different Ratations in Malignant Tissue

	Control	X Rays	Cobalt 60	3 ⁹ MeV Photon	30 MeV Electron	35 MeV Electron
Mitotic Count Mean \pm S D	392 \pm 27	89 \pm 09	154 \pm 21	914 \pm 19	251 \pm 20	267 \pm 29
Mitotic Loss Mean \pm S D		294 \pm 36	239 \pm 48	179 \pm 46	141 \pm 47	110 \pm 56
RBF		1	0.81 \pm 0.19	0.61 \pm 0.17	0.48 \pm 0.17	0.38 \pm 0.20
Pathol Mitoses per cent	80	40.0	15.0	6.0	2.0	1.0

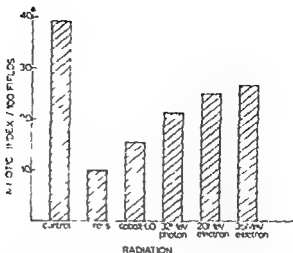


Fig. 1

Mitotic activity of controls and the other groups after different radiations in Walker-cancer

DISCUSSION

To compare the mitotic indexes (Fig. 1) obtained in the present work with the results of irradiation of normal degenerative tissue the same point of time i.e. 12 hours after irradiation was chosen for taking the samples. This time is suitable for the mitotic index for the control index was fairly high in this tumour tissue (39.2 ± 2.7). The mitotic indexes of the irradiated groups were lower than the control index and higher than in an earlier study, but the fluctuations were similar in that mitoses were fewest in number (9.9 ± 0.9) after X-rays and most numerous after 32 MeV electrons and the result was almost the same (22.1 ± 2.0) with 20 MeV electrons.

The effect of different types of irradiation on mitotic activity differed (Fig. 1). It can be said that the mitotic index grows with the energy of the radiation both with roentgen gamma rays and with the electrons used. Secondly it must be noted that pathological mitoses are most numerous in the X-ray and 32 MeV photons groups and significantly fewer in the groups given cobalt and both electrons (Table 1).

It is obvious that different types of irradiation at a certain time in this case within 12 hours of the irradiation exert differing effects. One such effect is the changing of the mitotic cycle: cell division is lengthened as Albert *et al.* (1960) established.

It is possible that the lengthening of the cell division is manifested differently in the irradiation groups and the result is a change in the mitotic cycle which is enough to produce great differences in rapidly growing tissue. The mitotic figures after roentgen and cobalt irradiation may correspond with results as late as 12 hours after the irradiation, whereas the effect of higher energy photons and electrons is

manifested as a prolongation of the mitotic cycle and thus as an ostensible increase in the mitotic figures

The pathologic mitoses established in all the irradiation groups are typical irradiation effects and their variations in the different groups are considerably greater than in the corresponding groups in normal regenerative tissue (Voutilainen *et al* 1967) as the growth rate is completely different. The more precise reasons for this require further study.

After subtracting the mitotic indexes of each irradiation group from the mitotic index for the control group and comparing the result with the difference for the X ray group by giving as the ratio the difference of the X ray group/difference of the irradiated group the figures in Table 1 were obtained as the RBE value for the X ray group. The other RBE values were smaller.

The RBE of high energy radiation was smaller in all the groups than in the earlier work. RBE was 0.81 ± 0.19 in the group given cobalt irradiation compared with 0.90 ± 0.26 for normal regenerative tissue. It was 0.48 ± 0.17 in the 20 MeV electron group and 0.52 ± 0.22 in the earlier study. These values were fairly similar in both investigations and the RBE value for cobalt was slightly higher than that arrived at by Lindén (1964) in his Walker cancer study. The RBE value obtained with 35 MeV electrons extremely low, only 0.38 ± 0.20 compared with 0.64 ± 0.31 in the earlier work. The difference was fairly great.

The RBE values at some other time may differ because of the possible variations in the mitotic index and thus the values obtained at a given time do not warrant any final conclusions. To be able to confirm the RBE values by administering different types of irradiation to the tumour tissue the post irradiation period before examination should be changed and a study should be made of the occurrence of mitoses as a function of the interval between the time of irradiation and the moment at which the biopsy specimen is taken.

SUMMARY

Three month old CB stock white rats with Walker cancer were used for the experiments. The tumour was implanted under the skin of the interscapular region. The irradiation was given 10 days later. The tumour dose was 300 rads and dose rate 40 rad/min. The radiation was given by 200 kV X rays (HVL 1.6 mm Cu), cobalt 60, 32 MeV photons of betatron and both 20 MeV and 35 MeV electrons of betatron. The irradiation time was at 7 p.m. and the animals were decapitated 12 hours after irradiation. Mitoses from the tumour were counted per 100 fields over an area of 0.785 sq mm.

The mitotic counts were highest in the control group and lowest in the X ray group. The RBE values for the different irradiation groups ranged from 1 to 0.38 when the RBE of the X ray group was 1. The

RBI of cobalt irradiation was 0.81 ± 0.19 of 20 MeV electron 0.48 ± 0.17 of 30 MeV electron 0.39 ± 0.20 and photons 0.61 ± 0.17

It seems that different types of irradiation have different effects at a certain point of time in the present work 12 hours after the irradiation. One reason obviously is the different changes in the mitotic cycle and lengthening of cell division.

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RECURRING DIGITAL FIBROUS TUMOR OF CHILDHOOD

1 Clinical and Morphological Aspects of a Case

By

JOHAN AHLQVIST PIIRKKO POHJANPELTO LARS HJELT and KARI HURME

Received 20 vii 66

A group of fibrous tumors with several features in common and called recurring digital fibrous tumors of childhood was recently described by Reye (1965). These often multiple and sometimes congenital tumors occurred in fingers and toes of children under 4 years of age. The growth of the tumors was comparatively slow but they recurred unless radically removed. The feature that distinguishes them from other fibrous tumors is the presence of cytoplasmic inclusion bodies. This observation led Reye to suggest virus etiology. As far as we know this tumor has not been reported outside Australia.

This paper describes a case diagnosed in Finland. The results of the virological studies on the tumor tissue will be dealt with in a subsequent paper (Pohjanpelto *et al* 1967).

CLINICAL PICTURE

The patient, a girl, was born to healthy parents in May 1965 in Hammenlinna, southern Finland. The pregnancy and labors were uneventful. There were two older healthy children in the family.

A reddish spot was said to have been present on the third digit of the left hand at birth. Two months later this spot started to grow and a similar spot appeared on the fifth digit of the same hand. At 4 months the distal part of the third finger had been converted into an erythematous tumor and a small biopsy was taken. After the biopsy the tumor started to grow rapidly, the spot on the fifth finger swelled and an erythema appeared at the tip of the fourth finger. In spite of a rather benign biopsy report, the clinical picture at 6 months prompted surgical treatment and the two distal phalanges of the third and the fifth finger were amputated together with the erythematous slightly swollen distal part of the fourth digit. A month later the stump of the third finger became erythematous, started to swell and reamputation had to be performed. Meanwhile the pathologists had become familiar with the report of Reye (1965) and at 8 and 9 months tissue removed from erythematous scars and from a rarified area in the bone close to the tip of an amputated digit was sent for virological examination.

MORBID ANATOMY

General Description

The amputation specimen from the large tumor in the third digit

Aided by a grant from the Sigrid Juselius Foundation



Fig. 1

The proximal part of the amputated third digit which was converted into a tumor. The distal interphalangeal joint and the nail are visible. Scale in millimeters. H&E.

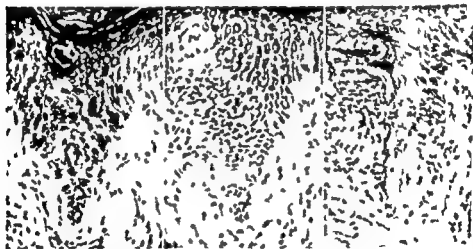


Fig. 2

Alterations present at the epidermal dermal junction at the site of the amputation specimens in which actual tumor was not yet present (H&E).

is shown in Fig. 1. The extent of the process can be seen since the distal interphalangeal joint and the nail are visible.

In the fourth finger which clinically was erythematous and slightly swollen the rete ridges of the epidermis were elongated and surrounded by lymphocytes and histocytes. The epidermal alterations seemed to start with an intracellular (probably cytoplasmic) vacuolation. The rete



Fig 3

Left Tumor tissue similar to the one on the dorsal aspect of the digit in Fig 2 Hematoxylin van Gieson *Right* Tumor cells from the same section at higher magnification

ridges seemed later to loosen up into a reticulum containing, in its meshes cells with small condensed nuclei (Fig 2 left to right). In the deeper parts of the specimen around Krause corpuscles associated with myelinated nerves there were strands of cells and fibrils which resembled nerves but contained no myelin sheaths.

On the volar side of the third digit (Fig. 1) there were alterations resembling those in fourth digit described above. On the dorsal and lateral aspects there was a highly cellular tissue reaching from the epidermis to the bone. The tissue was intimately associated with the bone. In Fig 1 the tissue actually erodes the bone under the proximal part of the nail. The elongated fibroblast like cells and collagen bundles formed interdigitating bundles (Fig 3 left). The tumor cell nuclei were rather large with 1-2 small nucleoli and a delicate chromatin network without any prominent margination of the chromatin (Fig. 3 right). Some regular mitotic figures were seen. Some tumor cysts and rather many mast cells were present in the tumor tissue.

In the original report on the specimens to the clinician the tumor was said to bring processes such as dermatofibromas, neurofibromatosis and fibromatoses into mind and we did not arrive at a definite

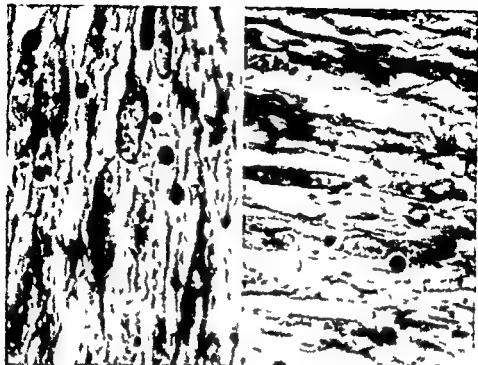


Fig. 1

Inclusions in sections from the tumor overstained with toluidine blue. In the right picture one round dark inclusion seems to have formed an impression into the nucleus.

diagnosis until Reve's report appeared. Until then we had seen no inclusions.

Inclusions

In sections stained with our H&E a few faintly eosinophilic inclusions were seen in the tumor tissue. Only formalin fixed and paraffin-embedded material was available. When the intensity of the eosin was increased the number of inclusions seemed to increase. The inclusions stained orthochromatically with strong solutions of toluidine blue (Fig. 1). They were easily distinguished from other tissue components in sections overstained with eosin, briefly washed in water and dilute acetic acid and thereafter stained in a strong, neutral solution of toluidine blue followed by rapid dehydration. In such sections the inclusions had a dark center surrounded by a rose rim.

The inclusions did not stain for 12 glycol groups with Schiff's reagent after oxidation with periodic acid. If not stated otherwise the following histochemical tests have been adapted from methods dealt with by Pearse (1960). The inclusions showed no alcohol stable metachromasy with toluidine blue. In a primitive "toluidine blue extinction test" (0.001 per cent solution in citrate buffers at pH 4.5 and 6 and

in tris buffers at pH 7 ■ and 9 staining time 18 hours at 4 °C examination in the staining solution) mast cells stained well nuclei nucleoli and the cytoplasm of the tumor cells poorly at pH ■ All these structures stained well at pH 4 but the inclusions did not stain well until at pH 7 in tris buffer In this solution almost all structures were metachromatic the metachromasim was not alcohol stable They gave a fairly strong tetrazonium reaction with Fast Blue B at pH 9.2 They did not stain with Scarlet Red for lipids With Sudan Black B they gave a weak positive reaction weaker than the reaction of for instance erythrocytes

The inclusions did not give a positive Feulgen reaction With a methyl green pyronine reaction carried out according to *Burch* (1966) the inclusions were not found to be pyroninophilic in contrast to the nucleoli and the cytoplasm of the tumor cells The orthochromatic basophilia of the inclusions was not noticeably influenced by digestion with ribonuclease (1 mg/ml at 37 °C for 2½ hours) or by extraction with 10 per cent perchloric acid for 16 hours at 4 °C These treatments removed the cytoplasmic basophilia of the tumor cells and of plasma cells in control sections

Some of the larger inclusions bore some resemblance to red blood cells but unlike the latter they did not stain for benzidine peroxidase

COMMENTS

Clinical Picture

Both clinically and morphologically the case corresponds to the cases described by Reye as recurring fibrous digital tumors of childhood

As far as we know this is the first case described outside Australia this seems to indicate that the tumor is not confined to Australia It is possible that it once recognized will not prove to be extremely uncommon as has been the case with so many other tumors

If these tumors are not extremely uncommon and if they continue to grow for a long time unless radically removed it seems that they should constitute a recognized syndrome in older patients Is it possible that they may undergo spontaneous regression? It would be interesting to obtain information about cases which have been left untreated

Morphology

In our case the process seemed to start with an erythema that corresponded morphologically to certain alterations in the epidermis and the upper dermis In these parts there were no clear inclusions nor any reticular degeneration of epidermal cells but it still seems that these alterations may be of some significance According to Reye the tumor tissue was separated from the epidermis by layer of normal collagen

Our studies suggest that the inclusions consist at least for the major

part of protein. Carbohydrate containing compounds and lipids are probably present in very small amounts if at all. The material seems to contain relatively few acidic groups. According to Reve the inclusions were pyroninophilic probably containing RNA but we have not been able to confirm the presence of RNA by any of the tests performed and it thus seems that in our case the inclusions either do not contain RNA or that they contain it in such a form that we cannot demonstrate it.

SUMMARY

A case diagnosed as recurring digital fibrous tumor of childhood is reported from Finland. The process may possibly start with certain alterations in the epidermis or at the epidermal dermal junction. Histochemical tests do not indicate the presence of RNA in the tumor cell inclusions.

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RECURRING DIGITAL FIBROUS TUMOR OF CHILDHOOD

2 Isolation of a Cell Transforming Agent

By

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Received 22 xi 66

The presence of inclusion bodies in the cells of recurring digital fibrous tumors of childhood led Reye (1965) to suggest that these tumors might have a virus etiology. From the recurrent tumor of a case described in part 1 (Ahlqvist et al 1967) of this study a cell transforming agent has been isolated.

METHODS

The pieces of tissue sent for virological examination were ground with sterile sand in a mortar centrifuged at a low speed and the supernatant was inoculated into cultures of primary human fibroblasts and continuous human amnion cells U cells (Pohjanpelto 1961). The cultures were incubated at 34 C and 37 C the medium for fibroblasts was Eagle's medium containing 3 per cent horse serum and 5 per cent tryptone broth and that for U cells Eagle's medium containing 5 per cent calf serum.

OBSERVATIONS

In the U cell cultures inoculated with the specimen cells detached from the glass in one to two weeks but in the following passages no such effect was observed. In cultures of fibroblasts areas of tightly packed cells of characteristic fibroblast morphology in random array developed. These areas grew in thickness and were transformed into thick clumps of cells visible to a naked eye. Later the clumps were often detached from the glass leaving holes which soon filled with newly formed fibroblasts. The clumping effect could be transferred to new cultures of fibroblasts suggesting that the agent responsible for it was able to multiply in primary human fibroblasts. So far 10 successful passages have been made using either a 1:10 dilution or concentrated supernatant as the inoculum. The response of the cells to infection varied to some extent. Sometimes focuses of rounded cells resembling epithelial cells appeared at the initial stage before the formation of clumps (Fig 1). Sometimes the whole cell layer increased in thickness

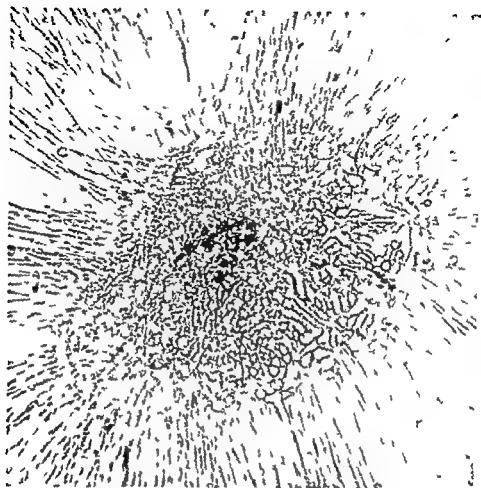


Fig. 1

Focus in a culture of primary human fibroblasts infected with the cell transforming agent

showing microscopically a characteristic pattern of alternate thick and thin areas (Fig. 2). Usually there was a marked change in the colour of the indicator in the medium during the first few days after inoculation as compared to the control showing increased acidity apparently due to increased cellular metabolism.

To obtain an idea of the size of the cell transforming agent medium from over the infected cells was collected daily, stored at -70°C , filtered through a Jena glass sinter G₃ able to retain bacteria and concentrated about 20 fold by drying in a vacuum. The filtration through a bacterial filter did not destroy the cell transforming ability of the agent indicating that it is smaller than bacteria and is possibly a virus.

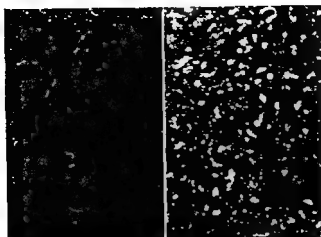


Fig. 2

Primary human fibroblasts uninfected left and infected with the cell transforming agent right

DISCUSSION

It is not likely that the cell transforming agent isolated from the tumor is a laboratory pick up because isolation from the original specimen was made three times. The small size of the agent shown by the filtration experiments indicates that it may be a virus. In our present state of knowledge we do not know whether it is the cause of the tumor but the ability of the agent to transform cells is consistent with this idea.

SUMMARY

An agent passing through bacterial filters and able to transform primary human fibroblasts *in vitro* has been isolated from a case of recurring fibrous digital tumor of childhood.

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INVESTIGATIONS ON ANIMAL *STAPHYLOCOCCUS AUREUS* STRAINS

2 Antigens

By

JEAN LOUIS MARANDON and PER OEDING

Revised 1 13 x 66

Relatively few studies have been made on the antigenic structure of animal *Staphylococcus aureus* strains. The results indicate that *Staph aureus* strains of human and animal origin have shared antigens but that there are quantitative as well as qualitative differences. Frequently the only criterion that the strains examined were true animal strains has been their isolation from animals or their surroundings, whereas the identity has not been ascertained by biochemical tests.

In a previous paper (8) a material of coagulase positive animal *Staph aureus* strains was examined and classified by biochemical methods. Strains producing beta hemolysin but not fibrinolysin were considered to be true animal staphylococci and were selected for serologic study. In the present report these animal strains have been examined for the presence of human *Staph aureus* antigens.

MATERIAL AND METHODS

The material consisted of 170 *Staph aureus* strains classified as animal strains (8). Eighty-four per cent of the strains were isolated from bovine mastitis, the others from animals other than the cow.

Agglutination was performed on slides using live bacteria and 11 human factor sera according to the method of Oeding. For technical detail see Hjalstal (2).

Agar gel diffusion was performed as described by Haukenes & Oeding (2). Eighteen live nutrient agar cultures of the strains were tested against 10 immune sera of human *Staph aureus* strain Wind 46 263 and Cowan 1. Lysate harite A poly saccharide 263 and protein A were included as references (12, 4).

RESULTS

Human *Staph aureus* agglutinogens. Seventy-eight (61 per cent) of the animal strains were typable in factor sera against human *Staph aureus* agglutinogens (Table 1). Forty strains did not agglutinate in any factor serum whereas two strains agglutinated spontaneously. The typable strains had a wide range of antigenic patterns. Their content of human staphylococcal agglutinogens seemed to be somewhat lower, 59 of the

strains had only one or two of the antigens and the agglutinations were often weak. The *h* antigen was the commonest. It was found in 30 strains which agglutinated strongly in *h* serum (Table 2). In 21 strains the *h* antigen was found alone.

TABLE 1
Agglutination of 120 Animal Strains in 13 Human Staph. aureus Factor Sera

	No	Strains	%
Typable	78		65
Not typable	40		33
Spont. aggl.	2		2

TABLE 2
Distribution of Agglutinogens in the 78 Typable Strains

Agglutinogen	No	Strains	%
a ₁	6		8
a ₂	14		18
b ₁	5		6
c ₁	19		24
e	4		5
h ₁	1		1
h	30		39
i	17		21
k ₁ k	4		5
m	3		4
n	2		3
*63-1	4		5
*63-2	19		24

TABLE 3
Human Staph. aureus Group Precipitinogens Present in 120 Animal Strains

	No	Strains	%
Polysacch. A	103		86
Polysacch. *63	5		4
Protein A	54		45

Human Staph. aureus precipitinogens Table 3 illustrates the distribution of three human *Staph. aureus* group precipitinogens in the 120 animal strains. One hundred and three strains gave a strong precipitation line against serum Wood 46 corresponding to standard polysaccharide A. Polysaccharide 263 was only demonstrated in five strains.

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INVESTIGATIONS ON ANIMAL *STAPHYLOCOCCUS AUREUS* STRAINS

2 Antigens

By

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Received 13 x 66

Relatively few studies have been made on the antigenic structure of animal *Staphylococcus aureus* strains. The results indicate that *Staph aureus* strains of human and animal origin have shared antigens but that there are quantitative as well as qualitative differences. Frequently the only criterion that the strains examined were true animal strains has been their isolation from animals or their surroundings whereas the identity has not been ascertained by biochemical tests.

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Agar gel diffusion was performed as described by Haukenes & Oeding (2). Eighteen hr live nutrient agar cultures of the strains were tested against white immune sera of human *Staph aureus* strains Wood JF 263 and Cowan I. Polysaccharide A (1), polysaccharide 263 and protein A were included as references (12, 4).

RESULTS

Human *Staph aureus* agglutinogens. Seventy-eight (65 per cent) of the animal strains were typable in factor sera against human *Staph aureus* agglutinogens (Table 1). Forty strains did not agglutinate in any factor serum whereas two strains agglutinated spontaneously. The typable strains had a wide range of antigenic patterns. Their content of human staphylococcal agglutinogens seemed to be somewhat low as 59 of the

were obtained by *Pillet et al* (10) and *Vercier et al* (9) who used the three Cowan sera for typing. *Valik & Singh* (7) and particularly *Live & Nichols* (6) were able to type a high percentage of their animal strains in human factor sera. *Grun* (1) and *Pulverer* (11) concluded that animal strains were much less often typable in human factor sera than human strains and if typable they had fewer and weaker agglutinogens. The fact that a number of strains are untypable would indicate that animal staphylococci have agglutinogens not represented in the human typing set. One new antigen has been demonstrated in this investigation. *Grun* (1) described a new agglutinogen (I) in an animal strain and this antigen was also demonstrated in human strains. Investigations by *Ishii* (5) and *White et al* (13) also indicate that animal staphylococci have specific antigens in addition to the agglutinogens shared with human strains. For type differentiation of animal staphylococci and their distinction from human strains it is important that more work be done on the specific animal antigens.

The examination of our animal strains for the presence of human *Staph aureus* group precipitinogens showed that they have the same type of antigenic ribitol teichoic acid as the cell walls of human strains. Beta linked N acetylglucosaminyl residues (polysaccharide A) were regularly demonstrated whereas alpha linked N acetylglucosaminyl residues (polysaccharide 263) seemed to be infrequent in our material. The wall also have protein A.

With regard to group precipitinogens as well there is no principal difference between animal and human *Staph aureus*. These antigens seemed however to be less readily demonstrable in the animal strains either because they are not present in all strains or because the quantities are small. In human *Staph aureus* strains the group precipitinogen show great quantitative variations and absorptions have not been carried out to exclude the presence of small amounts in the animal strains which appeared to lack the antigens.

SUMMARY

One hundred and twenty animal *Staph aureus* strains which had been isolated mainly from bovine mastitis pus and were identified biochemically have been examined serologically. Sixty five per cent of the strains were typable in human *Staph aureus* factor sera. The typable strains had fewer and weaker agglutinogens than human strains. The animal strains also had the same group precipitinogens as human staphylococci. A specific antigen was demonstrated. Animal *Staph aureus* strains therefore seem to have many of the same antigens as human *Staph aureus* strains although in smaller amounts and in addition certain specific antigens.

whereas 34 strains produced lines which corresponded with protein A. An interesting result was that 11 of these strains exhibited only a single line and not the double protein A line regularly observed in human *Staph aureus* strains against Cowan I immune serum. The line corresponding to that produced with human serum could not be demonstrated in these strains. When however crude protein A was prepared from two of the strains both antigens were present.

The majority of the animal strains also gave other non identified precipitation lines with the three human *Staph aureus* immune sera.

No correlation was found between human staphylococcal phage groups and the presence of group precipitins. It was however remarkable that 13 out of the 17 strains in which polysaccharide A was not demonstrated were not typable by phage.

Specific animal *Staph aureus* antigens. Rabbit immune sera were produced against three representative animal strains in which none of the human *Staph aureus* agglutinogens had been demonstrated. The unabsorbed sera agglutinated all our human *Staph aureus* type strains. The sera diluted 1:10 were then absorbed with representatives of the human type strains in order to remove all known human agglutinogens. After absorption none of the human type strains agglutinated in the three sera. Two of the absorbed sera had also been exhausted for antibodies against the homologous strains whereas the third serum still agglutinated the homologous strain and 16 other animal strains. After concentration of this serum an unknown precipitation line was produced on agar gel against the homologous strain and 12 of the animal strains which had been positive on agglutination.

DISCUSSION

The 120 strains which have been submitted to serological investigation are independent and according to biochemical tests true animal strains (8). However they are mostly bovine strains isolated from mastitis pus and therefore not representative of animal strains in general. The typing with human *Staph aureus* factor sera shows that specific human agglutinogens are also quite widely distributed in animal strains. It is possible that certain human *Staph aureus* agglutinogens are more frequently encountered in animal strains than others. Thus antigen *h* was quite frequent and strong in our material. But the results also show that there is a clear difference between animal and human strains with regard to the content of human agglutinogens. While nearly 100 per cent of human *Staph aureus* strains are typable in the set of human factor sera only 63 per cent of this material of animal strains could be typed. In addition the typable animal strains generally have weaker agglutinations and had fewer agglutinogens.

Other authors have been more or less successful in their attempts to type animal strains with human *Staph aureus* sera. Very poor results

Statens Seruminstitut · Pneumococcus Department · Copenhagen · Denmark

A NEW PNEUMOCOCCUS TYPE

Type 12A

By

ERNA LUND and AGNETE MUNKSGAARD

Received 18 x 66

The number of types given in the latest publication on serological classification of pneumococci is 81 (Lund 1962) a new type 12A is described here. In May 1965 an organism 411/65 was isolated from the blood of a 75 years old man. This strain manifested the usual cultural characteristics of a *Pneumococcus* and gave distinct capsular reactions in diagnostic pneumococcal sera (Lund 1963) of both type 12 and type 46 and moreover a weak reaction in type 44 serum. Further 3 strains giving similar reactions were subsequently isolated at Statens Serum Institut.

6 months after isolating the first of these organisms two strains of pneumococci which reacted in sera of types 12, 44 and 46 were sent to us by bacteriologist David Hansman¹ (Sydney) and during the following months 7 more similar strains were received from Australia giving a total of 14 strains.

These 14 strains were found to be Gram positive diplococci, soluble in bile, sensitive to optochin, growing aerobically as smooth colonies on 5 or 10 per cent blood agar showing α hemolysis and growing homogeneously in serumbroth.

SENSITIVITY

Tested by the tablet method (Lund 1965) the 14 strains showed identical sensitivity to antibacterial drugs being fully sensitive to penicillin, sulphonamides, tetracyclines, erythromycin, bacitracin, chloramphenicol, novobiocin, methicillin and nitrofurantoin; moderately sensitive to streptomycin, neomycin and fusidic acid and resistant to polymyxin and colimycin.

VIRULENCE

3 of the strains 411/65, 89 6/65 and 11743/65 showed high virulence to mice.

BIOCHEMICAL REACTIONS

None of the 14 strains fermented arabinose, xylose, dulcitol, sorbitol, mannitol or inulin in 8 days. All strains fermented glucose (with gas), galactose, lactose, sucrose, maltose, aesculin and salicin on the first day (Lund 1949).

¹ Our best thanks to bacteriologist David Hansman, Pathology Department, The Women's Hospital, Sydney, Australia, for the strains and for good collaboration.

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only react in the omni serum pooled serum E group serum 12 and factor serum 12c. In Table 2 it is seen that type 12 reacts in the same sera except in factor serum E but instead it reacts in factor serum 12b.

CROSS ABSORPTION

Cross absorption tests between the types 12, 44, 46 and the new type 12A (Table 3) showed that type 12A was so closely related to type 12 that it was practical to put both types into one group 12 (pn 12 + pn 12A).

TABLE 3
Cross absorption of the Types 12, 12A, 44 and 46

sera	antigenic formulas of the sera	capsular titers of the sera with types			
		12	12A	44	46
12 not absorbed	12a 1 b 1 ^o d	512	256	16	0
12 absorbed with 12A	12b	16	0	4	0
12 absorbed with 44	12a	128	198	0	0
12 absorbed with 46	12a 12b 1 ^o d	198	198	8	0
12A not absorbed	12a 12c 1 ^o d	256	512	32	16
12A absorbed with 12	12c	0	16	0	8
12A absorbed with 44	12a 12	198	256	0	16
12A absorbed with 46	12a 12d	256	256	16	0
44 not absorbed	44a 44b 1 ^o b 12d	32	32	256	0
44 absorbed with 12	44a 44b	0	0	198	32
44 absorbed with 12A	44a 44b 12b	16	0	198	16
44 absorbed with 46	44a 1 ^o b 12d	16	8	64	0
46 not absorbed	46a 1 ^o c 1 ^o d	0	32	32	16
46 absorbed with 12	46a 1 ^o c 1 ^o d	0	16	16	16
46 absorbed with 12A	46a 44b	0	0	0	128
46 absorbed with 44	46a 12c	0	16	0	64

type antigenic formulas

12	12a 1 ^o b 12d
12A	12a 12c 1 ^o d
44	44a 44b 1 ^o b 1 ^o d
46	46a 1 ^o c 44b

From the cross absorption tests the antigenic formula for type 12A can be given as 12a 12c 12d but the establishment of this new type makes it necessary for the formulas for three of the previously known types to receive an additional antigen (Table 3). The antigen 12d has been added to types 12 and 44 and 12c to type 46.

Type 12	12a 12b 1 ^o d
Type 44	44a 44b 12b 1 ^o d
Type 46	46a 12c 44b

ANTIPNEUMOCOCCAL SERUM

Antipneumococcal serum was prepared from the original strain 411/65 by immunizing ten rabbits (*Iund* 1960). The strain 411/65 gives capsular reactions and agglutination in unabsorbed sera of pneumococcal types 12, 44 and 46 but not in sera of the remaining 78 types. The titers of the reactions are given in Table 1.

TABLE 1

Cross Reactions (Capsular Titers) of the Pneumococcal Types 12, 12A, 44 and 46

unabsorbed sera	capsular titers with strains			
	12	12A	44	46
12	512	256	16	0
12A	256	512	32	16
44	32	32	512	32
46	0	32	32	128

Three batches of type 12 serum showed identical cross reactions and nearly the same titers (capsular and agglutination) with the new type 12A. Two batches of type 44 serum and two of type 46 also had identical cross reactions with type 12A. The most potent sera of the types 12, 44 and 46 were chosen for the cross absorption tests. No cross reactions other than those given in Table 1 were found in the sera mentioned.

DIAGNOSTIC SERA

A diagnostic serum is made specific (for the type group or pool) by absorption with the heterologous types. Entering the new type 12A into the system of diagnostic sera, types 12 and 12A are gathered into group 12, the diagnostic serum for type 12 reacting with both types in this group (Table 2).

TABLE 2

Diagnostic Pneumococcal Sera Reacting with Group 12 (12, 12A and 12A)

diagnostic sera	capsular titers with strains	
	in 12	in 12A
group 12	64	32
pooled serum F	32	32
omni serum	16	16
factor serum 12b	16	—
factor serum 12c	—	16

The diagnostic sera for the types 44 and 46 must be absorbed with type 12A to be typespecific. After absorption a strain of type 12A will

only react in the omni serum pooled serum F group-serum 12 and factor serum 12c. In Table 2 it is seen that type 12 reacts in the same sera except in factor serum c but instead it reacts in factor serum 12b.

CROSS ABSORPTION

Cross absorption tests between the types 12 44 46 and the new type 12A (Table 3) showed that type 12A was so closely related to type 12 that it was practical to put both types into one group 12 (pn 12 + pn 12A).

TABLE 3
Cross Absorption of the Types 12 12A 44 and 46

sera	antigenic formulas of the sera	capsular titers of the sera with types			
		12	12A	44	46
1 not absorbed	12a 12b 12d	512	256	16	0
12 absorbed with 12A	12b	16	0	4	0
12 absorbed with 44	12a	128	128	0	0
12 absorbed with 46	12a 12b 12d	128	128	8	0
12A not absorbed	12a 12 12d	256	512	32	128
12A absorbed with 12	12c	0	16	0	8
12A absorbed with 44	12a 12c	128	256	0	128
12A absorbed with 46	12a 12d	256	256	16	0
44 not absorbed	44a 44b 12b 12d	32	32	256	32
44 absorbed with 12	44a 44b	0	0	128	0
44 absorbed with 12A	44a 44b 12b	16	0	128	16
44 absorbed with 46	44a 12b 12d	16	4	64	0
46 not absorbed	46a 12c 12d	0	32	32	128
46 absorbed with 12	46a 12c 12d	0	16	16	128
46 absorbed with 12A	46a 44b	0	0	16	128
46 absorbed with 44	46a 12	0	16	0	64

type antigenic formulas

12	12a 12b 12d
12A	12a 12c 12d
44	44a 44b 12b 12d
46	46a 12c 44b

From the cross absorption tests the antigenic formula for type 12A can be given as 12a 12c 12d but the establishment of this new type makes it necessary for the formulas for three of the previously known types to receive an additional antigen (Table 4). The antigen 12d has been added to types 12 and 44 and 12c to type 46.

Type 12	12a 12b 12d
Type 44	44a 44b 12b 12d
Type 46	46a 12c 44b

TABLE 4

*Danish Type Designations of 82 Pneumococci
Antigenic Formulas According to Kauffmann and Iund*

type	antigenic formulas	type	antigenic formulas
1	1a	20	20a 20b 7g
2	2a	21	21a
3	3a	22	22a 22b
4	4a	22A	22a 22c
5	5a	23	23a 23b 18b
6A	6a 6b	23A	23a 23c 15c
6B	6a 6c	23B	23a 23b 23d
7	7a 7b	24	24a 24b 24d 7h
7A	7a 7b 7c	24A	24a 24c 24f
7B	7a 7d 7c 7h	24B	24a 24b 24c 7h
7C	7a 7d 7f 7g 7h	25	25a 25b
8	8a	27	27a 27b
9A	9a 9c 9d	28	28a 28b 16b 23d
9f	9a 9b 9c 9f	28A	28a 28c 23d
9\	9a 9b 9c	29	29a 29b 13b
9\	9a 9c 9d 9h	31	31a 20b
10	10a 10b	32	32a 27b
10A	10a 10c 10d	32A	32a 32b 27b
11	11a 11b 11c 11g	33	33a 33b 33d
11A	11a 11c 11d 11e	33A	33a 33b 33d 20b
11B	11a 11b 11f 11g	33B	33a 33c 33d 33f
11C	11a 11b 11c 11d 11f	33C	33a 33c 33e
12	12a 12b 12d	34	34a 34b
12A	12a 12c 12d	35	35a 35b 34b
13	13a 13b	35A	35a 35c 20b
14	14a	35B	35a 35c 29b
15	15a 15b 15c 15f	35C	35a 35c 20b 42a
15A	15a 15c 15d 15g	36	36a 9c
15B	15a 15b 15d 15e 15h	37	37a
15C	15a 15d 15e	38	38a 25b
16	16a 16b 11d	39	39a 10d
17	17a 17b	40	40a 7g 7h
17A	17a 17c	41	41a 41b
18	18a 18b 18c 18f	41A	41a
18A	18a 18b 18d	42	42a 20b 35c
18B	18a 18b 18c 18g	43	43a
18C	18a 18b 18c 18e	44	44a 44b 11b 12d
19	19a 19b 19d	45	45a
19A	19a 19c 19d	46	46a 12c 44b
19B	19a 19c 19c 7h	47	47a 34a 3 b
19C	19a 19c 19f 7h	48	48a

The formulas valid up to now are given for comparison (Kauffmann Iund & Eddy 1960)

Type 12 12a 12b

Type 44 44a 44b 12b

Type 46 46a 44b

All 14 strains of type 12A gave identical capsular reactions in the sera used for the cross absorption tests mentioned in Table 3. The stronger antigens in these 14 strains must therefore be identical.

Types 12A and 46 have been found to be highly virulent to mice whilst types 12 and 44 show only moderate virulence (Lund 1950). Types 12A and 46 have the antigen 12c in common but it is missing from types 12 and 44 it is therefore possible that this antigen is responsible for the higher virulence.

TABLE 5
14 Strains of Type 12A from 13 Patients

strain	patient	years of age	isolated from	clinical diagnosis
1 411/65	♂	76	blood	bronchopneumonia septicæmia
2 597/66	♀	67	blood	bronchopneumonia septicæmia
3 552/65	♀	69	blood	abscessus cerebri bacteriæmia
4 559/66	♂	71	blood	pneumonia duplex bacteriæmia
5 8956/65	♂	67	sputum	bronchopneumonia
6 11136/65	♂	70	conjunctiva	bronchit chron
7 11743/65	♂	■	sputum	bronchit chron
8 11793/65	♂	76	sputum	bronchit chron
9 12486/65	♂	54	sputum	bronchit pneumonia
10392/65				
10 10394/65	♂	61	clavicula meninges	osteomyelitis meningitis
11 6412/66	♂	46	sputum	bronchit chron
12 6365/66	♂	82	sputum	bronchit. chron
13 7197/66	♂	22	sputum	bronchit chron

The sources of the strains are given in Table 5. Most of the strains were isolated from elderly patients and predominantly from men of 11 male patients only three were less than 60 years of age and the 2 women were both in their sixties.

One of the strains (11136/65) was isolated from a conjunctival swab taken prior to operation on a cataract in a patient with chronic bronchitis. One patient died from meningitis caused by Pn 12A (strain 10394/65) following a motor accident the same type (strain 10392/65) having been isolated from an osteomyelitis of his clavicle.

SUMMARY

A new pneumococcal type 12A is described bringing the total number of types to 82. The new type reacts with the types 12, 44 and 46. It is put up as type 12A forming a group 12 with type 12.

14 strains of type 12A from 13 patients have been isolated in Denmark and Australia

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PRODUCTION OF CHLAMYDOSPORES BY *CANDIDA CLAUSSENI*

By

J. BROWN THOMSEN

Received 14 XII 66

A new species *Candida clausseni* was created by Lodder & Kreger van Rij in 1952 based on the micro and macromorphological deviations from their description of *Candida albicans* (1952). Like Stelling Dekker (1931) they found that the physiological reactions of the strain (received in 1910 from Claussen in Copenhagen labelled *Saccharomyces pastorianus*) was in agreement with those of *Candida albicans*.

The morphological characteristics on which the species was created were the absence of ball like clusters of blastospores on the pseudomycelium the incapability of producing chlamydospores a more elongated form of the blastospores after cultivation in milk extract and further the pseudomycelium never develops into the agar as it does in *Candida albicans*.

van Uden *et al* (1956/58) published some pathogenicity experiments with the type strain of *Candida clausseni*. They found that its pathogenicity was of the same order as that of *Candida tropicalis*. Without further comments they described a negative fermentation of galactose (a negative galactose fermenting ability was also mentioned in a previous report by the same authors in 1956) and in their quotation of the species description by Lodder & Kreger van Rij (1952) they wrongly reported the galactose fermentation as negative.

A negative galactose fermentation is also reported by Kocłova Kratochvilova *et al* (1963) in their Table 1 which gives a review of common and distinguishing features of *Candida albicans*, *Candida stellatoidea* and *Candida clausseni*. In their Table 3 and in a later report (Kocłova Kratochvilova 1964) the fermentation of galactose by *Candida clausseni* is reported as being positive. In 1967 the Central bureau voor Schimmelcultures still described the galactose fermentation of the type strain as positive (Slooff personal communication).

Sanchez Marroquin (1958) found further similarities between *Can*

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I wish to thank the Czechoslovak collection of Microorganisms (CMI SAS) for the strains generously supplied and the Central bureau voor Schimmelcultures for the type strain.

Candida albicans and *Candida clausenii* as to antibiotic sensitivity, the reduction of sulphates and the lack of hydrolysis of urea and tyrosine and concluded that these species may prove to be synonymous.

Arlagaveytia Allende & Legnani (1962) and *Arlagaveytia* Allende (1965) accepted the synonymy with reference to previous investigations by the above mentioned authors. Fragner (1962) still accepted *Candida clausenii* as a separate species with mere reference to the publication by Lodder & Kreger van Rij (1952).

In all of the publications on *Candida clausenii* previously mentioned it is accepted that this species is not able to form chlamydospores¹.

This paper describes the observation of chlamydospore formation in *Candida clausenii* experiments on the serum tube formation ability and some physiological properties.

MATERIALS AND METHODS

Strains Four strains of *Candida clausenii* were used. Three one cell cultures were made from each strain using the technique of Orskov (1952). The properties of the four original strains and of the twelve cloned cultures were alike, three of the original cultures being the type strain of the species.

The four strains were the following:

1 Type strain of *Candida clausenii* CBS No 1949

From the Czechoslovak collections of Microorganismus

2 (CHI SAS) 29-31-1 = IFO 0759/1961 = CBS 1949

3 (CHI SAS) 29-31-2 = CBS 1949

4 (CHI SAS) 29-31-3

During the experimental period the strains were kept in our collection on malt extract agar slants and transferred every third month.

Chlamydospore formation Two media (a and b) were used to promote the formation of chlamydospores.

a Rice medium with the following composition (Taschdjian 1953)

White rice	20 g
Special agar noble (Difco)	20 g
Distilled water	1000 ml

b Same composition as medium a with Tween 80 in a 1 per cent concentration (Taschdjian 1957)

The cultures were scratched into a thin layer of the medium on slide covered with cover glasses and cultivated in a wet chamber (Petri dish) at room temperature which during the experimental period was 20-25° C. The culture was examined on these media daily for 8 days.

Fermentation The media were made principally according to the direction of Lodder & Kreger van Rij (1952) with the following composition of the basic sugar free substrate:

Yeast extract (Difco)	14 g
Water	2520 ml

The sugars were added to a final concentration of 2 per cent. The sugars were heat treated for 2 x 20 min at 100° C (Lodder & Kreger van Rij 1952) used 15 min.

¹ At the Third International Symposium on Yeasts in Bratislava July 1966 Dr Wickerham (Peoria Illinois U.S.A.) informed me that he too had observed chlamydospores of the same form as described in this paper in a strain of *Candida clausenii*.

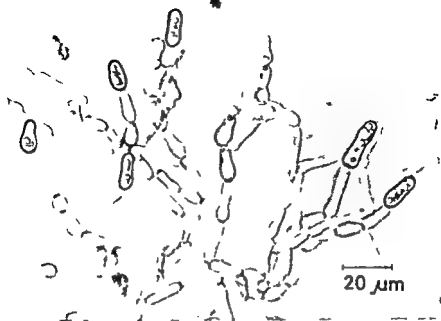


Fig 1

Candida clausenii: Chlamydospores in Rice medium *a*

at 120 °C) Table 1 shows the various sugars used. Two different makes of galactose and sucrose were used.

The production of gas was used as an indicator for the fermentation (Durham glasses).

The cultures were incubated at room temperature (20–22 °C). The reactions were read daily for 10 days. The inoculation was made by a tiny inoculum from malt extract agar cultures.

Assimilation of carbon sources. The reactions were performed on solid medium with the addition of yeast extract in plastic Petri dishes (not air tight) by the method used by Lodder & Kreger van Rij (1952).

With reference to Wickerham & Burton (1948) the carbon sources that were tested are shown in Table 2.

The reactions (growth zones) were examined after cultivation at 25 °C for 2–3 days and again after further cultivation for 2–3 days at 17 °C.

Rapid identification test for *Candida albicans*. The filamentation reaction (Reynolds & Braude 1956; Taschdjian *et al.* 1960; Stenderup & Brown-Thomsen 1964) was performed in human serum in small tubes. The serum was inoculated with yeast cells from a 2–3 day old malt extract agar culture (35 °C) and incubated for 48 hours at 35 °C. The yeast cells were then examined on slides for the presence of characteristic filaments. The activity of the serum was continuously checked by the use of a typical *Candida albicans* strain.

RESULTS

All cloned cells produced chlamydospores in approximately 2–3 days in medium *a* which did not contain Tween 80. Figs 1 and 2 show the

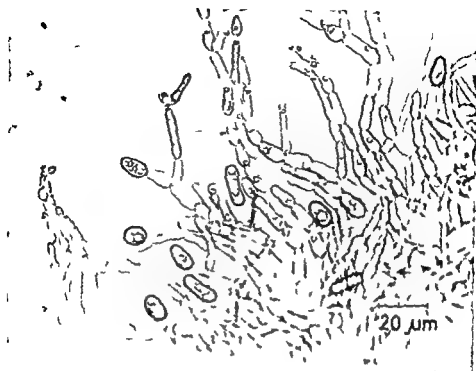


Fig. 2

Candida clausenii: Chlamydospores in Rice medium =

chlamydospores in this medium. In medium *b* which contained Tween 80 in a 1 per cent concentration extremely few chlamydospores were observed.

The results of the fermentation reaction are shown in Table 1.

TABLE 1
Fermentation Reaction

d-glucose	(Merck)	+
d-galactose	(Merck)	+
d-galactose	(Fluca)	+
d-maltose	(Merck)	+
d-sucrose	(Pflanzlich)	(+)
d-sucrose	(BDH Analar)	((+))
d-lactose	(Merck)	—
inulin	(Merck)	—
raffinose	(Merck)	—

One pair of brackets indicates that the Durham glasses could not be completely filled in 10 days.

Two pairs of brackets indicate that only a small bubble was formed.

The results of the assimilation reaction (auxanographic method) are shown in Table 2.

TABLE 2
Auxanogram

d-glucose	(Merck)	+
d-galactose	(Merck)	+
d-sucrose	(Pflanzstehl)	+
d-maltose	(Merck)	+
d-lactose	(Merck)	—
l-sorbose	(Merck)	+
d-cellobiose	(Sigma)	—
d-trehalose	(BDH)	+
d-melibiose	(Pflanzstehl)	—
raffinose	(Merck)	—
d-melezitose	(Merck)	—
inulin	(Merck)	—
d-xvlose	(Merck)	+
l-arabinose	(Merck)	+
d-arabinose	(Pflanzstehl)	variable
d-ribose	(Sigma)	—
erythritol	(Merck)	—
adonitol	(Merck)	+
dulcitol	(Merck)	—
d-mannitol	(Merck)	+
d-sorbitol	(Merck)	+
alfa methyl d glycosid	(BDH)	+
salicin	(G T Gurr)	—
lactic acid	(Merck)	+
succinic acid	(Merck)	+
citric acid	(Merck)	+
inositol	(Merck)	—

The filamentation reaction in serum was negative for all the strains. The control strain of *Candida albicans* produced typical filaments.

DISCUSSION

In the original description of the species *Candida claussonii* (Jodder & Kreger van Rij 1952) it was stated that the fermentation and assimilation reactions used for the description were like those for *Candida albicans* and that the properties characterizing *Candida claussonii* were the incapability of producing chlamydospores and the deviation from the accepted morphology of *Candida albicans* as mentioned in the introduction.

The author agrees as to the similarities between the fermentation and assimilation reactions of *Candida albicans* and *Candida claussonii* mentioned in the original description (where fermentation of glucose, sucrose, maltose and lactose and assimilation of glucose, galactose, sucrose, maltose and lactose were tested). With special reference to the diversities as regards the fermentation of galactose mentioned in the introduction it is worth noticing that fermentative utilization of lactose was positive for both strains tested (Table 2).

The chlamydospores produced by these strains of *Candida claussonii* are dissimilar to those normally seen in freshly isolated strains of *Candida albicans* but according to Wickerham (1935) and Jodder &

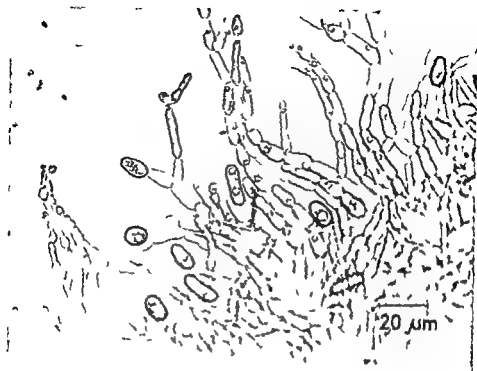


Fig. 2

Candida clausenii: Chlamydospores in Rice medium a

chlamydospores in this medium. In medium *b* which contained Tween 80 in a 1 per cent concentration extremely few chlamydospores were observed.

The results of the fermentation reaction are shown in Table 1.

TABLE 1
Fermentation Reaction

d-glucose	(Merck)	+
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d-maltose	(Merck)	+
d-sucrose	(Pflanzlich)	(+)
d-sucrose	(BDH Analar)	((+))
d-lactose	(Merck)	—
aulia	(Merck)	—
raffinose	(Merck)	—

One pair of brackets indicates that the Durham glasses could not be completely filled in 10 days.

Two pairs of brackets indicate that only a small bubble was formed.

The results of the assimilation reaction (auxanographic method) are shown in Table 2.

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3 per cent. Immediately after extraction the teeth were cut at a right angle to their length axis into two or three parts thereby obtaining a quicker and better fixation with the neutral formalin. Decalcification followed in formic acid sodium citrate solution. After double paraffin embedding the middle part of the root was sectioned at a right angle to the length axis of the tooth. 6-8 μ m thick sections and stained with haemalun eosin and periodic acid Schiff. Each type of staining was performed in the same batches including both diabetic and nondiabetic material.

Results In all the sections the peripheral third of the pulp showed only small vessels whereas the central part presented small and large vessels and nerves.

In the PAS stained sections from diabetics PAS positive mural thickenings were seen in both small and large vessels. These vascular changes seemed to be most pronounced in the central areas of the pulp but could be seen even in the peripheral part. Similar changes could not be demonstrated in the control material.

Calcifications in the pulp were found in diabetics and nondiabetics. But in diabetics these calcifications were more frequent and were often sickle shaped and embracing the vessels. In some areas the calcification was so marked that only remnants of vessels could be discerned.

Discussion The present investigation shows that patients with diabetes mellitus of long duration present late diabetic manifestations in the form of vascular changes in the dental pulp identical with those observed in other tissues i.e. marginal gingiva and periodontal membrane (Russell 1966, 1967). The higher frequency and peculiar shape of the calcifications in the diabetic dental pulp can hardly be connected with a contingent hypertension as they are often extramural.

The two groups are yet too small to allow any definite conclusions. In regard to the morphological variation of the calcifications an investigation to show how early and how often these alterations occur is in preparation.

Conclusion A preliminary investigation shows that in patients with diabetes mellitus of long duration late diabetic vascular changes may be demonstrated in the dental pulp which in these patients also seems to exhibit a specific type of calcification.

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THE ADOPTIVE TRANSFER OF EXPERIMENTAL MOUSE AMYLOIDOSIS BY INTRAVENOUS INJECTIONS OF SPLEEN CELL EXTRACTS FROM CASEIN TREATED SYNGENEIC DONOR MICE

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A number of investigations in recent years dealing with the pathogenesis of experimental amyloidosis has indicated the decisive role played by mesenchymal cells in particular by those belonging to the reticulo endothelial system (Teitum 1954 1956 1964 Cohen *et al* 1965) Observations of the histochemical events taking place during the induction of experimental amyloidosis together with studies of the influence of various enhancing and inhibiting factors on the dynamics of amyloid formation led to the concept of a biphasic local cellular formation of amyloid (Teitum 1964) In light of these premises it was considered a probability that the primary pyroninophilic phase of amyloid formation induced in one animal might be transferred to a second syngeneic animal with spleen cells thus without delay eliciting the second amyloid phase in the recipient Such experiments have proved successful thereby substantiating the crucial role of cells in the pathogenesis of experimental amyloidosis (Werdelin & Ranlov 1966 Ranlov & Werdelin 1967)

The following experiment aims at the elucidation of the nature of the factor responsible for the adoptive transfer of experimental mouse amyloidosis in a syngeneic system—in particular at the question whether its activity is related to the intact cell

MATERIAL AND METHODS

Animals Throughout the experiments the same stock of randomized closely inbred C3H mice 2-4 months of age were used Sex distribution was equal

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Experiment A

Donor mice 25 randomly selected were treated with 17 daily casein injections 5 times a week according to the method originally described by Lucynski (1959). Per injection 0.5 ml of a solution (5 per cent) of casein in 0.25 per cent NaOH was given subcutaneously in the back. This treatment resulted in minute amounts of amyloid in the spleens of approximately $\frac{1}{3}$ of the donors. In all donor spleens a heavy pyroninophilia was evident. The day after the last injection all donor mice were killed by cervical dislocation and the spleens were harvested and pooled in cold isotonic saline. From each spleen a small specimen was fixed in neutral formalin for histological examination. The total yield of splenic tissue was 6.5 gm (wet weight).

Cell suspension The spleens were gently teased apart in a loose fitting Potter-Elvehjem homogenizer. After filtration twice through a metal sieve the suspension was washed twice in cold isotonic saline and finally made up to a suspension containing spleen cells equivalent to 200 mg wet weight of splenic tissue per ml (corresponding to approximately 200×10^6 nucleated spleen cells per ml). 6 ml of this suspension (the equivalent of 12 gm wet weight of donor spleen) was kept for injection in the experimental group I (see below).

Cell homogenate and fractionations Extracts of donor spleen tissues were made largely according to the method for preparing antigenic tissue extract described by Medaure (1963). The remaining cell suspension corresponding to an initial wet weight of pooled donor spleens of approx. 50 gm was centrifuged for 5 minutes at 2°C at $1500 \times G$. The supernatant saline was discarded and the cells resuspended in 10 ml cold isotonic saline to which 30 ml icecold distilled water was thereupon added under vigorous stirring. The cells now sticky and osmotically swollen were spun down again at $1500 \times G$ (2°C) cut up coarsely with scissors resuspended in 40 ml of icecold distilled water and spun out again. The gelatinous cell mass was cut up again with scissors resuspended in 17 ml distilled water and in volumes of 8.5 ml homogenized in a Waring blender at half speed for 5 minutes followed by top speed for further 2 minutes. After the removal of air bubbles the homogenate was finally exposed to ultrasonic irradiation for 45 seconds using an MSE, Ultrasonic Disintegrator frequency 70 kc/s output 60 W. The final result was 17 ml of a transparent yellowish fluid. In order to restore the original salt concentration 0.081 volume 2 M NaCl was added. This caused a heavy precipitate to form which was thereupon removed by centrifugation at $5000 \times G$ for 10 minutes. This sediment will in the following be referred to as "DNA protein" while the corresponding supernatant will be referred to as "crude semisoluble extract" (Medaure 1963).

The sedimented fraction termed "DNA protein" was suspended in isotonic saline so that 0.5 ml of the suspension contained material representing an initial wet weight of 100 mg of the pooled donor spleens from which the preparation started. The final dry weight of "DNA protein" in 0.5 ml of this suspension was determined as 70.25 mg (after evaporation of water and deduction of the 4.5 mg NaCl). The dry "DNA protein" was microscopically an amorphous highly Feulgen positive material without recognizable cell structures. It may be considered made up of all the cellular DNA DNA linked enzymes and histones (Klenow 1966).

The supernatant fraction termed "crude semisoluble extract" was likewise adjusted so that 0.5 ml represented an initial wet weight of 100 mg pooled donor spleen. The final dry weight in an aliquot of 0.5 ml was 2.5-3.0 mg. This dry material may be considered derived from mitochondria, microsomes, various soluble enzymes and more than 90 per cent of the cell RNA (Klenow 1966).

Treatment of Recipients

Group I 12 mice were given intravenously in the tail 0.5 ml of the original suspension of viable spleen cells from the casein treated donor mice. This dose corresponds to an initial wet weight of 100 mg pooled donor spleen and to approx. 100×10^6 nucleated spleen cells. The trypan blue test showed 85-90 per cent viable cells in the inoculum. In this and in all the following groups including the controls heparin was added so that 0.5 ml contained 10 IU.

Group II 15 mice received intravenously 0.5 ml whole homogenate (that is "crude semisoluble extract" + "DNA protein") corresponding to an initial wet weight of 100 mg pooled donor spleen. 0.5 ml of this inoculum contained 45.55 mg dry material.

Group III 14 mice were given intravenously 0.5 ml suspension containing nuclear fraction (DNA protein) corresponding to an initial wet weight of 100 mg pooled donor spleen

Group IV 14 mice received intravenously 0.5 ml of the suspension containing the cytoplasmic fraction (crude semisoluble extract) corresponding to an initial wet weight of 100 mg pooled donor spleen

Within 2 hours after transfer each mouse in every group received the initial dose of 0.03 mg nitrogen mustard subcutaneously (Krasol 100%) The nitrogen mustard treatment was repeated after 48 hours 4 days after transfer all mice were killed by ether inhalation In the meantime no mortality occurred

Control Groups

Donor mice 20 normal, randomly selected mice were killed and their spleens pooled in cold isotonic saline Spleen cell suspensions and extracts were prepared exactly in the manner described above

Recipients

Group I (corresponding to the experimental group I) 8 mice were given intravenously 0.5 ml of a suspension of spleen cells from normal untreated syngeneic donors This dose corresponded to 100 mg initial wet weight of pooled donor spleen and to approx 100×10^3 nucleated cells Viability was 80 per cent

Group VI (corresponding to the experimental group III) 10 mice received intravenously 0.5 ml of a suspension of DNA protein corresponding to an initial wet weight of 100 mg pooled normal donor spleen

In the controls treatment with nitrogen mustard and time of killing were identical with those of the experimental groups No spontaneous mortality was observed

An additional experiment under similar conditions Experiment B was designed in order to investigate 1) the histological events taking place during the first few days after transfer of DNA protein derived from spleens of casein treated donors with and without subsequent treatment of the recipients with nitrogen mustard 2) whether amyloidosis similarly could be induced by subcutaneous administration of "DNA protein" from casein treated donors and 3) whether the observed amyloid inducing potency of the DNA protein from casein sensitized donors might be influenced or even abolished by treatment of the inoculum with DNase prior to injection

Experiment B

In Experiment B inbred randomized C3H mice of the same stock as those employed in Experiment A were used The treatment of the donors and the preparation and the fractionation of spleen cell extracts were identical to the procedure described in Experiment A

After 17 days of casein treatment spleens from 25 randomly selected donors were harvested and pooled The DNA protein derived from this pool was washed with isotonic saline to a suspension containing the equivalent of 10 wet weight of pooled donor spleen per 0.5 ml 28 mice each received 0.5 ml of this suspension intravenously in the tail 14 of these mice groups 1-3 (Table I) were subcutaneously treated with nitrogen mustard as described above and killed 4 days after transfer The remaining 14 mice comprising groups 4-7 (Table I) were treated likewise except that they did not receive nitrogen mustard (Table I) 8 mice each were given 0.5 ml of the same DNA protein subcutaneously in the back followed by nitrogen mustard treatment and killed after 4 days

An amount of the DNA protein equaling 600 mg wet weight of pooled donor spleen was washed and resuspended in 0.02 M phosphate buffer (pH 6.5) with 0.001

TABLE 1

Experiment 1. Number of animals developing amyloidosis and their mean degrees of spleen amyloidosis 4 days after the transfusion of whole viable spleen cells or various fractions of spleen cells from either normal mice or mice previously treated with sodium caseinate for 17 days

Group	Pretreatment of donors	Number of recipients in group	Treatment of recipients				Mean degree of recipient spleen amyloidosis
			Transfused material	Nitrogen mustard injections (0.05 mg)	Recipients killed after	Number of recipients developing amyloidosis (of total)	
I	17 daily injections of sodium caseinate	12	Whole viable spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	12/12	+ + + (2-4)
II		15	Whole homogenate of spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	15/15	+ + + (2-4)
III		14	Nuclear fraction ("DNA protein") of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	14/14	+ + + (2-4)
IV	"	14	Cytoplasmic fraction (crude semisoluble extract) of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/14	0 (0-0)
V	None	8	Whole viable spleen cells i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/8	0 (0-0)
VI	"	10	Nuclear fraction ("DNA protein") of spleen cell homogenate i.v. (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/10	0 (0-0)

Experiment II Number of animals developing amyloidosis and their mean degree of pleen amyloidosis 1, 2 or 4 days after the transfusion intravenously or subcutaneously of nucleoprotein (DNA protein) derived from either normal mouse pleen cells or spleen cells from donors previously treated with vitamin B₁₂ for 17 days

I	17 daily injections of vitamin B ₁₂ caseinate	5	Nuclear fraction (DNA protein) of spleen cell homogenate 1% (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Day 1	24 hour	0/5	0 (0-0)
II		5		Day 1	4 days	0/5	0 (0-0)
III		4		Days 1 and 3	4 days	4/4	+ + (2-3)
4		5		None	24 hours	0/5	0 (0-0)
5		4			2 days	1/4	(+) (0-2)
6		5			4 days	2/5	(+) (0-2)
7		8	Nuclear fraction (DNA protein) of spleen cell homogenate subcutaneously (dose = the equivalent of 10 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	1/8	0 (0-0)
8		6	DNA treated nuclear fraction (DNA protein) of spleen cell homogenate 1% dose = the equivalent of 100 mg wet weight of pooled donor spleen	Days 1 and 3	4 days	6/8	+ + (2-3)
9	None	11	Nuclear fraction (DNA protein) of spleen cell homogenate 1% (dose = the equivalent of 100 mg wet weight of pooled donor spleen)	Days 1 and 3	4 days	0/11	0 (0-0)

Al MgCl so that 0.5 ml of the suspension contained DNA protein equivalent 100 mg wet weight of pooled donor spleen. This suspension was thereafter incubated for 2 hours at 20°C with 40 microgramme of beef pancreas DNase D6C4 (Worthington). After incubation 0.5 ml was injected intravenously into each of 11 recipients which subsequently received the usual course of nitrogen mustard until killed after 4 days. They comprised the group 8 (Table 1).

Finally group 9 comprised the controls corresponding to group VI of Experiment A (Table 1). 11 mice received DNA protein intravenously from normal untreated donor spleens derived from 15 randomly selected donors (dose = the equivalent of 100 mg wet weight of pooled normal donor spleen). After the usual course of nitrogen mustard the recipients were killed after 4 days.

Histology

Tissues were fixed in neutral formalin, paraffin embedded and sections were cut 5 microns thick. The following stains were employed: Haematoxylin in the P.A.S. technique, the Feulgen stain and alkaline Congo red. Amyloid was identified by morphology and birefringence with Congo red under crossed polars. The individual degree of amyloidosis was estimated semiquantitatively on sections of spleen and graded from 0-6 according to Christensen & Hjort (1959). In Table 1 mean values are expressed as pluses.

RESULTS

The main results of the present experiments are outlined in Table 1. It will be seen that no amyloid at all could be found in mice receiving viable spleen cells or DNA protein derived from spleens of normal untreated donors (Experiment A, groups V and VI; Experiment B, group 9)—a total of 29 recipients. In contrast viable spleen cells, whole spleen homogenate and DNA protein of spleen cells derived from casein treated donors were equally effective in inducing amyloidosis in the respective recipients, while mice receiving crude semisoluble extract from the same pool of casein treated donors failed 100 per cent to do so. Moreover in Experiment A the amyloid developing as a result of the various treatments apart from a 100 per cent positivity showed a striking degree of uniformity in the 3 groups receiving viable spleen cells, whole spleen extract or DNA protein respectively (Figs 1-3). This uniformity points towards the DNA protein fraction as the agent responsible for amyloid induction in the recipients.

From the results of Experiment B (Table 1) it appears that animals receiving DNA protein from casein treated donors did not develop recognizable amounts of splenic amyloid before at least 2 days after the transfer (groups 1-6). While in group 3 4 out of 4 recipients of DNA protein subsequently treated with nitrogen mustard developed amyloidosis when killed after 4 days, only 2 out of 5 recipients in group 6 not receiving nitrogen mustard did so when similarly killed after 4 days. This indicates that although nitrogen mustard markedly enhances amyloid formation in the recipients it is not completely necessary for the initiation of the amyloidogenic process in this system.

From the observations in group 9 (Table 1) it is apparent that subcutaneous administration of the DNA protein to recipients subsequently treated with nitrogen mustard was nearly ineffective in inducing



Fig 1 Section of spleen from mouse treated with an intravenous injection of viable spleen cells from casein treated syngeneic donors. The injection was followed by a short course of nitrogen mustard and the recipient was killed 4 days after cell transfer (experiment 4 group I). Spleen amyloidosis grade 3 Haematoxylin eosin $\times 35$

Fig 2 Section of spleen from mouse treated with an amount of whole spleen homogenate from casein treated syngeneic donors equivalent to 100 mg pooled wet weight of donor spleen killed after 4 days of nitrogen mustard (Experiment 4 group II). Showing spleen amyloidosis grade 3 Haematoxylin eosin $\times 35$

amyloidosis. Only 1 out of 11 such treated mice responded with amyloid formation and only to a very slight degree.

Finally, all mice injected intravenously with D₁₀Ase treated D₁₀A protein from casein treated donors followed by the usual course of nitrogen mustard (group 8) developed splenic amyloid to exactly the same degree as did mice similarly treated with intact D₁₀A protein from the same pool.

Morphologically, the amyloid deposits were identical to those usually seen in experimental mouse amyloidosis. They were predominantly arranged as perifollicular rings in the spleens. The amyloid substance was moderately eosinophilic and weakly PAS positive except for the most peripheral parts where new formed amyloid showed a vivid purple—often in close relationship to PAS positive reticulo-endothelial cells. The amyloid substance stained pink with alkaline Congo red and exhibited the characteristic green birefringence when viewed under crossed polars. In all sections examined the amyloid substance was Feulgen negative. The whole amyloid deposition in livers and kidneys was sparse or absent.



Fig 3 Spleen from mouse similarly treated with nuclear fraction (DNA; protein) from the same pool of casein treated syngeneic donors Nitrogen mustard and time of killing as above Spleen amyloidosis grade 3 (Experiment A group III) Haematoxylin eosin $\times 35$

Fig 4 Spleen from mouse similarly treated with cytoplasmic fraction (crude semi soluble extract) from the same pool of casein treated syngeneic donor spleens Administration nitrogen mustard treatment and time of killing as above (Experiment A group IV) No detectable amyloid resulted from this treatment Haematoxylin eosin $\times 35$

DISCUSSION

That the transfusion of syngeneic spleens or their homogenates *per se* are not responsible for the initiation of the very rapid amyloidogenic process in the recipients of our transfer system seems evident from the negative results obtained with spleens from untreated donors in the present and in earlier experiments (Werdelin & Rantow 1966). Thus it seems that only spleen cells or subcellular fractions of spleens from hyperimmunized donor mice are capable of initiating the rapid amyloidogenic process in syngeneic recipients. Furthermore, immunization (casein treatment) of the donor mice prior to cell transfer must endure at least 2 weeks of daily antigenic treatment (Werdelin & Rantow 1966). The necessity of these conditions must be emphasized owing to the not infrequent findings of splenic hyaline in such conditions as wasting disease or homologous disease attributable to graft *versus* host reactions in mice. In these conditions the injections of immunologically competent allogeneic spleen cells into immunologically incompetent weanling F₁ hybrid mice may be followed 2-4 weeks later by a fatal wasting disease. Among other pathological features this syndrome may

include the deposition in the splenic perifollicular regions of a homogeneous eosinophilic material which undoubtedly is amyloid (Fiscus *et al* 1962). In such conditions where the recipient F₁ hybrid spleen is populated by antigenically different parental strain cells the development of amyloid deposits in the course of a few weeks is to be expected in most strains of mice Barnes *et al* (1962) in lethally irradiated mice inadequately restored with syngeneic liver (fetal) or bone marrow described the development of secondary disease characterized by lymphoid aplasia hyperplastic granulopoiesis and from day 40 onwards wide spread replacement of splenic tissue with an eosinophilic substance later recognized as amyloid (Bradbury & Vicklem 1965). Provided an adequate protection with larger amounts of syngeneic lymphoid or bone marrow cells both the syndrome and the amyloid lesions were prevented. Undoubtedly these irradiated and insufficiently restored mice were suffering from a variety of infections which alone will account for the subsequent amyloidosis. These investigations too argue against any possibility of spleen cells *per se* being amyloidogenic when injected into adult syngeneic recipients. This is further supported by *in vitro* studies by Dutton (1966) who showed little or no proliferative response occurring in mixed cultures of syngeneic spleen cells from mice. In contrast a rapid increase occurred in allogeneic combinations.

In the present experiments only nuclear fractions were capable of initiating the amyloidogenic process in the recipients. As all of the antigenic activity of cells has been found to reside exclusively in the cytoplasmic fractions (Medawar 1963 Monaco *et al* 1965) there seems to be no reason to ascribe the amyloid inducing effect of our transfused material to antigenic properties—disregarding the syngeneic donor-host relationship.

From our results it further appeared that recipients subsequently treated with nitrogen mustard developed amyloidosis far more markedly than did untreated recipients of the same inoculum. Such an amyloid enhancing effect of nitrogen mustard was originally described by Teitelum (1954) however the exact nature of the intracellular events induced by the mustard are not well understood.

Among the effects of the various alkylating agents is an initial profound depression of the rate of incorporation of H³ thymidine into DNA (Wheeler 1956) (Hart *et al* 1964). A parallel depression in the RNA and protein synthesis (Hart *et al* 1964) though usually to a lesser extent (Bischoff & Busch 1961 Haring *et al* 1961 Hart *et al* 1964). From 36 to 48 hours after the administration of the mustard in our experiments employing normal murine as well as neoplastically transformed cells, there was a marked increase in DNA content and large secretions of RNA and protein (Hart *et al* 1961 Lewis *et al* 1963 Booth *et al* 1964). This occurs in parallel with a marked swelling of the cells to a volume 1.5 to 2 times that of untreated cells (Booth *et al* 1964). The mechanism of this swelling is not clear. Treatment of cells grown *in vitro* with a high dose of nitrogen mustard does not produce any measurable changes in the physical or chemical properties of the rDNA while DNA content increases with high concentrations of mustard however many physicochemical studies have shown a markedly reduced viscosity (Brewer & Irons 1963) with electron microscopic investigations

demonstrated the retention by DNA of increased amounts of protein unseparable by normal purification procedures (Colder *et al* 1964).

Recent experiments by Chanmougan & Schwartz (1966) showed that increased as well as inhibited antibody formation may follow treatment of rabbits with 6-mer captapurine. They proposed that nucleic acids released from the injured cells could account for the enhancement of the immune response in the former case.

The constant association of lymphoid aplasia with the various conditions in mice characterized by wasting although bacterial or viral infections may account for many of their features has occasionally revived interest in a possible trophic function of lymphoid cells (Iontil 1962 Barnes *et al* 1962). As the possible mediators of such activity have been specified DNA or its break down products. It has been shown that radioactively labelled DNA can be reutilized as such by other dividing cells in the animal for example by regenerating liver cells (Bryant 1962) or in skin grafts (Diderholm *et al* 1962). Polynucleotides may be incorporated directly into lymphocytes (Hill & Pospisil 1960) and strain L cells (Bensch & King 1961). The surprisingly long life span of lymphocytes as measured by their turn over of P-3 labelled DNA (Ottensen 1964) has been interpreted as the reutilization of large fractions of the nucleic acids or nucleoproteins of their predecessors (Hamilton 1958 Sundberg 1960). Trowell's (1957) observation of pyknotic remnants of phagocytized small lymphocytes within reticular cells made him submit a hypothesis of reutilization in a lymphocytic macrophage cycle (Trowell 1958) and modified versions of these views have later been expressed (Sundberg 1960 Berman 1966). Such a mechanism has been claimed to account for the transfer of immune reactivity by lymphoid cells between syngeneic individuals as being a transfer of self replicating nucleoprotein subsequently being reutilized as viable templates for antibody and/or protein synthesis (Hamilton 1958). Similarly a possible transfer of immune reactivity with nucleic acids or nucleotides between cells within the same individual has been proposed (Chanmougan & Schwartz 1966). However evidence seems largely circumstantial.

On the other hand purified preparations of DNA have been shown to be capable of transferring murine leukemia (Hays *et al* 1957) and various tumours (Laterjet *et al* 1958). DNA from adult mouse I cells has been found highly stimulatory of *in vitro* polypeptide synthesis in contrast DNA from mouse embryos or peritoneal tumour cells were nearly inactive (Holland & McLarthy 1964). Nucleic acids and enzymatically digested nucleic acids have been shown capable of enhancing antibody synthesis (Johnson *et al* 1963 Braun *et al* 1965). An extensive review of problems concerning the possibility of nucleic acid mediated genetic transformation has recently been published by Ledoux (1965). The bearing of these observations on the results of the present experiments rely mainly on the fact that the material with which our amyloidosis apparently was transferred contained large amounts of nucleoproteins—that is DNA or DNase treated DNA.

The transferred agent which in the present material seems responsible for the initiation of the amyloidogenic process in the recipients is not likely to be identical with the transfer factor originally described by Lawrence (1960). This factor which hitherto has been demonstrated in humans only is capable of transferring delayed hypersensitivity to normal recipients. Transfer factor itself appears to be a low molecular weight dialysable material (Lawrence *et al* 1962) it has been identified in a polynucleotide fraction and in a fraction containing various globulins and RNA both fractions derived from human white blood cells (Baran *et al* 1966). The search for such a transfer factor in animal species has as a whole not been successful. Assuming that such a factor akin to the transfer factor of Lawrence was operating in the present material it should be present in the supernatant fraction of the spleen cell homogenate which fraction on the contrary failed to induce amyloidosis in our recipient mice.

It is only reasonable to believe that the particles of our "DNA protein suspension when injected intravenously primarily ended up in phagocytizing cells of the recipients reticulo endothelial systems preferably in the spleens and livers which are known to be the primary sites of murine amyloid formation. Moreover this will account for the failure to induce amyloidosis with the same inoculum when administered subcutaneously.

The direct demonstration of the participation of reticulo endothelial cells in amyloid formation has been repeatedly demonstrated (Teitum 1954 1956 1964 Cohen *et al* 1965). From these and other investigations (Werdelin & Rantlov 1966 Rantlov & Werdelin 1967) it seems indisputable that the amyloidogenic process is mediated primarily by cells thus ruling out any speculations concerning circulating precursors etc. The two-phase cellular theory of local secretion (Teitum 1964) clearly points out the existence of two consecutive events in the mesenchyme: 1) A primary preamyloid phase of weeks duration characterized by increasing cytoplasmic pyroninophilia of reticular and plasmacytoid cell lines. 2) In the course of persistent antigenic stimulation the second amyloid phase gradually sets in characterized by the accumulation at the same sites of amyloid deposits in the immediate vicinity of large often P.A.S. positive reticulo endothelial cells. With the onset of the second amyloid phase the initial pyroninophilia gradually disappears. However the relationship between the pyroninophilic cell of the initial phase and the P.A.S. positive reticulo endothelial cell of the second phase undoubtedly synthesizing amyloid has never been clarified. Are they identical or at least do they represent the same cell? The latter being the result of differentiation of the former? In our earlier experiments in which pyroninophilic spleen cells from casein treated donors caused amyloid to develop in syngeneic recipients we felt justified in assuming that this was the case (Werdelin & Rantlov 1966 Rantlov & Werdelin 1967). However the results of the present experiments do

indicate their relationship as an indirect one involving an intermediate stage of cell death or cell fragmentation. On this account and within the limits of the biphasic cellular theory the following mechanism of experimental amyloid formation may be suggested:

1) During prolonged antigenic treatment an increasing stimulation of mainly cellular immune reactions occurs (Ranlov 1967, Ranlov & Jensen 1966). This hyperstimulation leads to increasing pyroninophilia of various reticular and plasmacytoid cells—the former probably representing the activated state (blast transformation) of lymphoid cells corresponding to the simultaneous disappearance of small lymphocytes in the perifollicular regions of the spleen. The pyroninophilia reflects the rapid increase in the production of various antibodies, nucleic acids, proteins, and enzymes necessary for cell division.

2) After a few weeks of persistent antigenic stimulation the capacity for further proliferation is used up and a state of exhaustion is reached. From now on the pyroninophilia will decrease and on the cellular level the dominating feature will be cell death or cell fragmentation. This is an intermediate stage and represents the so-called inversion phase (Teitum 1964).

3) The increasing cell death (or cell fragmentation) results in the release of various nuclear and cytoplasmic fragments, some of them self-replicable nucleic acids or nucleotides carrying some kind of information from their metabolically highly active parental cells. These fragments of nuclear material are locally phagocytized in RFS cells. Under the specific conditions of overloading the ingested (self-replicable) material will impose protein synthesizing information on the RFS cell, thus causing a profound disturbance in its function. This disturbance may either be a direct one, the information being imposed directly on the phagocytizing cell itself, or it may be an indirect one, the information being passed on to other reticular cells (nursing) with different potencies. The distinction between these possibilities on histological grounds is not facilitated in the light of evidence of the labile nature of the morphologic appearance of the various cells that are involved (Birman 1966). The visible results will be a cytoplasmic PAS positivity of reticular cells indicating intracellular synthesis of glyco proteins and extracellular deposition of amyloid.

SUMMARY

The adoptive transfer of experimentally induced amyloidosis between syngeneic mice is described. It appears that not only viable spleen cells but also the nuclear fraction of spleen cell homogenates derived from casein-treated donors are effective in initiating a rapid amyloidogenic process in healthy recipients when administered intravenously, while cytoplasmic fractions are not. Only spleens from donors pre-sensitized with casein were capable in these respects.

Of a total of 51 mice receiving viable spleen cells whole spleen extract nuclear fraction of spleen cells and DNase treated nuclear fraction respectively derived from casein treated donors all 51 mice developed amyloidosis to the same degree within 4 days while 14 mice receiving cytoplasmic spleen cell fraction derived from the same casein treated donors all were negative as were 7 out of 8 recipients treated with the "nuclear fraction subcutaneously

29 control mice receiving corresponding doses of spleen cells or spleen extracts from normal non sensitized donors failed 100 per cent to develop amyloid lesions In all cases including the controls a short course of nitrogen mustard treatment was given after the transfer

From other similarly treated groups subjected to serial killings after 1 2 and 4 days it appeared that amyloid did not develop in the recipients until 48 hours after the transfer

The results are taken as supporting a possibility of different cell types operating specifically for each of the two phases in experimental amyloid formation

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DNA SYNTHESIS IN FREE FULL SKIN AUTOGRAFTS IN MICE

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The take of a free skin autograft is comparable in many aspects to the closure of a surface wound by apposition of the skin edges. The epidermal mitotic activity plays a dominant part in the healing of the wound (Montagna 1962).

It has been established that free skin autografts get initial blood circulation at the time when the grafts lose their edema e.g. on the 4th or the 5th day after grafting (Clemmesen 1965). Other observations reveal that free skin autografts have a reduced blood circulation during the first 6 days after the grafting procedure and the blood circulation was determined active or normal on the 7th day (Marclmann 1965).

Once the blood circulation is established in skin grafts it should be possible to demonstrate epidermal deoxyribonucleic (DNA) synthesis in dividing cells with tritiated thymidine distributed by the circulation which has been demonstrated to be a reliable method of labelling dividing nuclei (Measier & Leblond 1960). We thought it might be of interest to evaluate the regeneration of the epidermal layer in skin autografts in accordance to incorporated tritiated thymidine during the initial phase of the healing process after grafting.

MATERIAL AND METHODS

Sixtyone female mice of a non homologous strain 4 weeks old and weighing about 20 to 30 grams each were used as experimental animals. The investigations were performed in two experimental groups of which the first was a preliminary.

A modified technique of free skin autografting in mice described earlier (Cross *et al* 1960) was used. Nembutal was administered intraperitoneally in a dose 100 mg per 10 grams of body weight. The mice were shaved dorsally. After application of an adhesive film onto the grafting area a 5 cm strip of surgical micropore tape was applied across the dorsum just anterior to the femoral insertion of the hind legs. A 1 x 1 cm piece of full skin was cut and the axis of the ear flaps was turned 180 degrees in regard to the bed. The graft was again fixed to the bed with surgical micropore tape.

An hour before killing the animals 2 μ c per gram of body weight of tritiated thymidine were injected intraperitoneally.

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Fig. 1

Completely necrotic transplantate 5 days after transplantation. Magnification 6×10 .

In the pilot experiment the mice were killed 4, 5 and 6 days after skin grafting and in the second group 4, 5, 6, 7, 9, 10, 11 and 19 days subsequently.

Histologic specimens were cut from the middle portion of the grafted area including the host-graft junctions on both sides of the graft as well as normal skin outside the grafted area. The histologic samples were fixed in 4 per cent formalin and cut into 4μ sections.

Autoradiograms were prepared by the dipping method with Ilford K 5 photoemulsion and 10 days exposure time was used. After development the preparations were stained with haematoxylin-eosin.

Transplantates in the tables have been divided in 3 groups:

- a) completely necrotic
- b) partly regenerated (regeneration foci in the middle or in the border areas of necrotic epidermis)
- c) completely regenerated (epidermis normal throughout the whole area of the transplantate)

Incorporation of ^3H thymidine in the premittotic cells has been used as regeneration indicator. Cells were considered labelled with grain count at least 5 times above the background.

RESULTS

Experiment 1

This experiment was performed to find a good grafting technique and the best postoperative period for investigating epidermal DNA synthesis in the grafted skin.

The experimental series included 14 mice. Table 1 shows that the epidermal layer of the grafted area had partly regenerated (= in accordance with incorporated tritiated thymidine) in one animal.

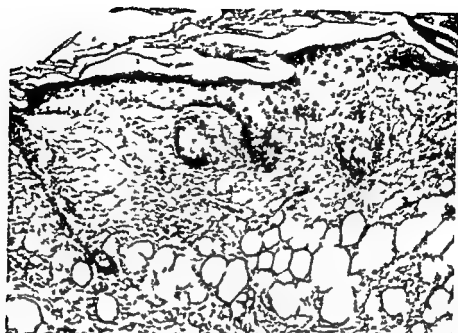


Fig. 2

Complete & regenerated transplantate 10 days after transplantation
Magnification 6×10

4 and 5 days after grafting. In animals sacrificed 11 days after grafting, a partial regeneration of the epidermal layer was noticed in all test animals.

TABLE 1

Regeneration of the Epidermal Layer in Free Full Skin Autografts in Accordance with Incorporated Tritiated Thymidine

Time after transplantation	Number of animals	No regeneration (completely necrotic)	Regeneration	
			Partial	Complete
4 days	7	6	1	0
5 -	3	2	1	0
6 -	4	0	4	0

Experiment 2

In this series the experiments in the first group were repeated and continued. This group included 47 test animals. The results are shown in Table 2.

An increased number of labelled cells were seen in grafts 7 days-old indicating epidermal regeneration. The epidermis was almost completely regenerated from the 10th day on after the grafting procedure and the regenerative process continued in an increasing manner 12 days after the grafting.

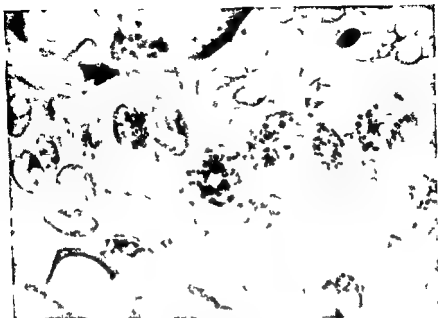


Fig 3

Completely regenerated transplantate 10 days after transplantation
Magnification 6×100

TABLE 2

Regeneration of the Epidermal Layer in Free Full Skin Autografts in Accordance with Incorporated Tritiated Thymidine

Time after transplantation	Number of animals	No regeneration (completely necrotic)	Regeneration	
			Partial	Complete
4 days	5	4	1	0
5 -	8	5	3	0
6 -	9	1	1	0
7 -	5	1	4	0
9 -	7	4	1	2
10 -	7	1	3	3
11 -	7	0	4	3
12 -	6	1	1	4

There were more activity in the edges of the grafts than in the central parts of the epidermal area. In some sections increased labelled DNA synthesis were noticed even in the central parts of the graft.

Vascular congestion and numerous leucocytes were seen in the dermis 4 to 11 days after grafting. Regenerating mesenchymal cells of the dermis were seen in the earlier groups before regeneration was observed in the epidermal layer.

DISCUSSION

The results reported reveal that increased epidermal DNA synthesis appears in cells of free full skin autografts in mice from the 7th postoperative day increasing at least until the 12th postoperative day.

These findings correlate well to earlier observations on the blood circulation in free skin autografts. According to Marchmann (1965) the grafted skin has a reduced blood circulation during the first 5 days postoperatively, whereas the circulation was considered active or normal on the 7th day. There was no or very low activity in the epidermal layer during the 4-5-6th day although the skin graft should have established anastomosis between the vessels. The first burst of mitotic activity appeared after the blood circulation can be considered normal (Brophy & Lobitz 1959; Clemmensen 1965; Marchmann 1965; Pinkus 1952).

We were not able, however, to determine the source of the labelled cells in the autografts, whether they were cells of the grafts themselves or cells migrating from the epidermal layer outside the graft. The experiments will be continued in this respect.

SUMMARY

The study comprises a total of 61 female mice divided into two test groups. An attempt was made to investigate the epidermal DNA synthesis in free full skin autografts with tritiated thymidine injected 4-5-7-9-10-11 and 12 days after the skin grafting procedure. The results revealed that a definite increased regenerative activity of the epidermal layer of the grafts, measured with incorporated tritiated thymidine, occurs on the 7th and until at least the 12th day postoperatively. The increased epidermal DNA synthesis correlates well to the established active blood flow that free skin autografts get on the 7th day after grafting.

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ELECTRONIC CELL COUNTING IN CELL CULTURE STUDIES

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Counting of cultured cells by the haemocytometer technique is time consuming, and the total error of the method has been estimated to some 9.5 per cent (Biggs & MacVillan 1948). Counting of erythrocytes by an electronic cell counter has been studied by Oulie (1959) who found a mean error of 0.85 per cent due to the apparatus and a total mean error (including sampling) of 2.1 per cent. These values correspond well to the results obtained for another similarly designed counter (Brecher *et al* 1956; Mattern *et al* 1957). Compared with the haemocytometer procedure the electronic counting thus provides more exact erythrocyte counts.

In order to evaluate the applicability of electronic counting in cell cultivation, Celloscope counts obtained from suspension cultures of P-388 cells have been analyzed.

MATERIALS AND METHODS

Cell Cultures

The cultures employed in this study were all suspension cultures of P-388 cells (Dawe & Potter 1957). The growth medium was a slight modification of Eagle's Minimum Essential medium (Eagle 1959). Methocel (Farle *et al* 1954) was added to a final concentration of 1.2 g/l and pluronic acid (Stum & Parker 1960) to 1.0 g/l. Phenol red was used as a pH indicator and penicillin (100 I.U./ml) and streptomycin (100 µg/ml) were added. To the medium was added 10 per cent of heat inactivated horse serum.

Counting Procedures

The Celloscope has been constructed mainly for erythrocyte determination. The erythrocytes are suspended in salt solution and pushed through a capillary tube by suction. At the same time an electric current is sent through the capillary tube. The passage of each erythrocyte induces the conductivity of the suspension to change. This is proportional to the cell volume. The cell suspension is so diluted that single cells pass the capillary tube. The change in resistance is amplified and the Celloscope is finally registered. The disc is then rotated and the next cell is counted. The minimum change of resistance (or minimum time) is recorded. As

¹ Celloscope Farle

& Co., Stockholm, Sweden

all particles exceeding a certain size will be registered the diluting medium should be filtered and its background count determined.

In the actual experiments a solution of 0.85 per cent NaCl was used. Several 1 ml volumes of this solution were run through the apparatus preceding each test to wash out cells and debris from previous tests. The background count of the saline was 2 to 4 (i.e. 0.5 to 2 per cent of the total count) and the culture samples were prepared by adding 20 ml of saline to 1 ml of cell culture. The dilution was prepared in an Erlenmeyer flask, transferred to a small beaker and the counting performed immediately. The preparation of the dilution would take 3 to 4 minutes. For each sample the counts of three separate 1 ml volumes were registered and the cell concentration was calculated from the mean of these sample counts. The whole procedure would take some 10 minutes.

The discrimination knob was used in position 40. According to the instruction leaflet which is concerned with erythrocyte counting, this would exclude cells with a volume less than $100 \mu^3$. Assuming that the P-388 cells have a roughly spherical form this would correspond to P-388 cells with a diameter of approximately 5μ .

Visual Counting in the Haemocytometer

Care was taken to avoid uneven distribution of the cells due to settling. Samples were taken with Pasteur pipettes and transferred undiluted to a Barker haemocytometer. The cells in 4 squares (each 1 mm^2) were counted and the highest and lowest values were disregarded. The mean of the three remaining values was registered as the cell count.

RESULTS

All experiments recorded except one were performed in 4 Erlenmeyer shake flasks. Two flasks (A and B) had serum from lot I and showed actively proliferating cells that appeared normal under the microscope. The two other flasks (C and D) were supplied with serum from lot II and contained cells which multiplied slowly and had a granular appearance. Accumulation of debris was seen in the medium of these latter cultures.

In experiment 3 samples were taken from a spinner flask. The cells were multiplying at a fair rate and showed no morphological signs of degeneration.

1. Exactness of the Apparatus

The mean error of Celloscope counts of erythrocytes has been found to be about one tenth of that observed with visual counting technique (Oulie 1959). The apparatus was tested for P-388 shake cultures in a similar way. Cell count registration was performed as described and the discrepancies between individual counts were compared to the corresponding variations obtained with visual counting technique.

The results indicate that within the same sample the Celloscope records values far more accurately (Table I).

The relation to variations in individual visual counts is of the same magnitude as that reported for erythrocyte counting.

With both methods there was a convincing trend towards a smaller variability with great cell density.

TABLE 1

Celloscope and Haemocytometer Counts Variations within the same Sample

Sample No	Celloscope values			Haemocytometer values		
	Mean	Range	Range/mean	Mean	Range	Range/mean
1	255	7	1.37	24	7	14.6
2	264	6	1.13	34	7	10.3
3	265	5	0.94	34	11	16.7
4	264	12	2.27	35	10	14.3
5	297	3	0.50	32	6	9.4
6	309	6	0.97	32	11	31
7	276	1	0.18	29	11	15.5
8	262	5	0.95	38	8	10.5
9	441	4	0.45	54	12	11.1
10	432	11	0.68	56	2	1.8
11	418	3	0.35	49	3	3.0
12	422	4	0.48	46	1	1.1
13	650	8	0.61	104	13	6.3
14	700	8	0.56	113	11	4.9
15	571	4	0.35	71	6	4.2
16	613	2	0.16	66	10	7.6
			\bar{x} 0.75			\bar{x} 8.37

P 388 cells cultivated in suspension were counted. The Celloscope values are the numbers registered on the electronic counter; the haemocytometer values express the cell density in the culture (cell $10^4/\text{ml}$).

Range presented as \pm per cent of mean.

2 Evaluation of the Sampling Error

Variations in the counts for one sample as well as between samples from the same culture have been recorded in Table 2. Although the variations between counts from different samples were on the whole greater they seemed to be within the range expected within a single sample.

TABLE 2

Sampling Error of Celloscope Method

Variations within the sample				Variations within the culture					
Sample	Mean	Range	Range/mean	Culture	Mean	Range	Range/mean		
A1	334	2	0.3	A	310	9	1.5		
A2	322	2	0.4						
B1	340	2	0.3						
B2	335	2	0.4	B	331	13	2.1		
C1	372	0	0.0						
C2	371	4	0.6						
D1	363	2	0.3	C	371		0.3		
D2	361	2	0.3						
			\bar{x} 0.3						
							\bar{x} 1.02		

Variations in individual units within a sample and between samples from the same culture of P 388 cells cultivated in suspension. Cultures A and B were well growing; cultures C and D partially senescent.

Range presented as \pm per cent of mean.

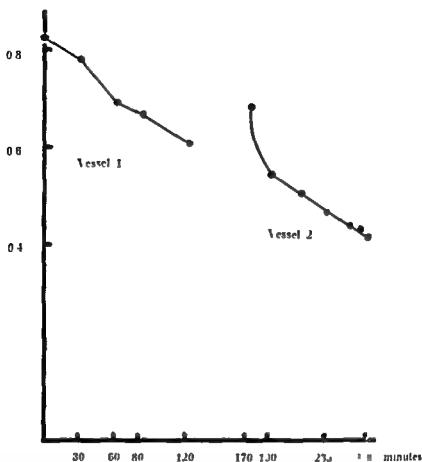
Cell count 10^4 

Fig. 1

Celoscope counts as a function of time interval from sampling and sample dilution to cell count registration. Two samples of 10 ml were harvested at (1), diluted with 200 ml of 0.85 per cent filtered saline each and left at room temperature. Subsamples were counted at the times indicated on the figure. Preceding each counting the vessel was agitated to avoid settling of the cells. Each plot represents the mean of three individual counts.

3 The Effect of Time Interval from Sampling to Cell Count Registration

To 10 ml samples from a spinner flask (10) was added 200 ml of filtered 0.85 per cent NaCl and the diluted samples were left at room temperature in 1 liter Erlenmeyer flasks. Countings were performed at intervals through a period of 4½ hours. Preceding each counting, the 1 liter Erlenmeyer flask was gently rotated to avoid uneven cell-distribution. Twenty ml of the dilution was then transferred to a small beaker and counting performed. Fig. 1 shows that the cell count decreases after sampling.

and sample dilution. The decrease starts immediately and is fairly proportional with time.

4. The Effect of the Nature of the Diluting Medium

As the Celloscope counting procedure necessitates a dilution of the sample the effect of the diluting medium was tested. The growth medium (containing 3 per cent of horse serum) and 0.85 per cent filtered saline were compared. Each dilution was performed immediately before counting and cells from rapidly dividing and from more stationary cultures were counted after dilution in both liquids. The results are presented in Table 3.

TABLE 3
Effect of the Diluting Medium on Celloscope Counts

Sample source Flask	Counts obtained after dilution in	
	Saline	Growth medium
A	473	498
B	464	504
C	495	451
D	489	472

P 388 cells cultivated in suspension were counted. Cultures A and B were well growing cultures C and D partly degenerating. One ml samples were diluted with 99 ml of the respective diluting media: 0.85 per cent filtered NaCl and the growth medium (containing 3 per cent horse serum).

Mean of 3 individual counts. The range within each sample was of the magnitude of 2 to 10.

For both types of cultures the difference between parallel cultures was greater when growth medium was used for dilution. A difference was observed between well growing and stationary cultures: the former give higher values in growth medium whereas the latter presented the higher values when saline was used as the diluting medium.

5. Haemocytometer and Celloscope Counts from Rapidly Dividing and more Stationary Cultures

Two of the cultures tested showed signs of degeneration whereas in the two others no such signs could be detected. Cell counts were recorded for a period of four days. After two days the cultures were inoculated with fresh medium corresponding to a concentration of 1 or 2. Figure 2 presents the cell increase with time as determined by the two counting methods.

It is evident that the Celloscope counts and the haemocytometer counts would lead to different conclusions with respect to growth. The Celloscope cannot reveal the difference in growth rate by the usual technique as distinct differences were found.

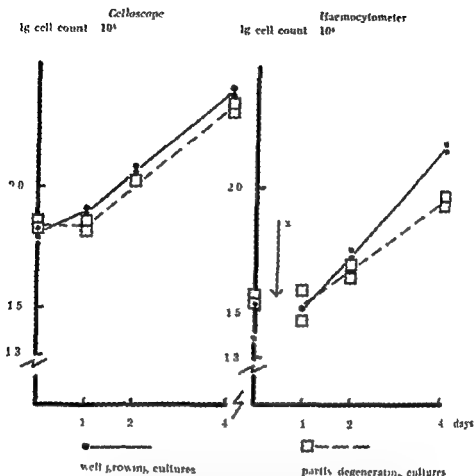


Fig. 9

The effect of cell culture condition on Celloscope and haemocytometer counts. Two well growing and two partly degenerating shake flask cultures of 1 358 cells were counted for a period of 4 days. Each plot represents the mean of three individual counts.

∧ Variations between parallel samples too great to allow reasonable exactness of the curves.

DISCUSSION

The present experiments apply to the reproducibility of the method as well as to technical procedures involved in preparation of the samples. Lastly the specificity of the apparatus as regards particle nature was tested.

For particle counting, in general the Celloscope is a very precise instrument. The results in Table I show that individual counts in the Celloscope vary far less than in the haemocytometer and that the Celloscope presents the greatest advantage when counting cultures with a low cell density. The relative exactness of the methods corresponds well to the values reported for erythrocyte counting, (Oulte 1959).

The data recorded to estimate the error introduced by sampling (Table 2) are too few to give more than purely tentative informations. However the sampling error was found to be relatively small. The total error (including sampling error) with Celloscope technique was far less than the variations within the same sample in the haemocytometer (Tables 1 and 2). An additional advantage is obvious: the Celloscope method eliminates the personal bias involved in visual counting.

However the counting of cultured cells presents special problems. The experiment concerned with the effect of the diluting medium indicates that this factor is of some importance (Table 3). In what way this factor affects the results cannot be evaluated from the present experiment. Because the difference between parallel cultures was greater after dilution in growth medium, saline was chosen for further tests. The choice was further based upon the claim that fibrous material is considered to be an undesirable contamination (Walter *et al.* 1957).

The time necessary for sampling and sample dilution greatly influenced the cell counts which decreased proportionally with time. One value not in agreement with this statement (Fig. 1 b) was probably due to instability of the apparatus: the range of the individual counts around this value was 94, ten times the average range value in this experiment. It is evident that saline also influences the counts, possibly by causing cell disintegration, and the time factor must be strictly defined before conclusions are attempted.

Fig. 2 shows that the Celloscope does not exclude particulate matter other than viable cells. With the present technique the Celloscope registers debris accumulation in the same manner as cell multiplication. This can also to some extent explain that the cell counts obtained with the Celloscope are higher than the visual counts from the same culture (Fig. 2).

It must be concluded that for particle counting the Celloscope *per se* is a far more precise tool than the haemocytometer. However the counting of cells propagated in culture presents special problems. The influence of the growth medium and the additional counts from sources other than viable cells must be controlled before the Celloscope can replace the haemocytometer in cell culture studies.

SUMMARY

- 1 Cell counting in cell culture experiments has been performed. Conventional visual technique and an electronic counter (Celloscope 101) have been compared.
- 2 The Celloscope gave individual counts which varied far less than the individual counts in the haemocytometer.
- 3 The Celloscope counts were influenced by the diluting medium and by the time lapse from sample dilution to cell count registration. These factors should be standardized.

- 4 The Celloscope method did not discriminate satisfactorily between rapidly dividing and more stationary cultures. Because of this fact the apparatus must be considered less suitable for cell culture studies until the method has been utterly refined.

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A NEW TISSUE CULTURE METHOD FOR STUDIES ON ANTIBODY SYNTHESIS IN VITRO BY LYMPHOID CELL SUSPENSIONS

By

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Tissue culture of lymphoid cells poses some special problems (cf *Trowell* 1965). It has none the less been extensively used for immunological studies (reviewed by *Stavitsky* 1961 *Ainus* 1964 *Nossal* 1966). A continuous antibody production lasting for some time *in vitro* has been demonstrated repeatedly with the use of lymphoid cells stimulated *in vivo*.

Lymphoid cells can be cultured separately as cell suspensions or in organ cultures as tissue fragments or slices. The former method has some advantages as pointed out by *inter al* *Vaughan et al* (1960) *La Via* (1962) *Michaelides & Loons* (1963) *Halliday & Garvey* (1964) and *Svehag* (1966). *Svehag* stresses the following points: a/ quantitative and qualitative uniformity of replicate cultures b/ uniformity of environment for all cells of a culture c/ uniform and rapid uptake of compounds incorporated into the medium d/ elimination of intercellularly retained antibody (p. 261). On the other hand cell suspensions have usually resulted in less antibody production than organ cultures have (see however conflicting views by *La Via* 1962 *Dresser* 1966). Antibody production has usually been demonstrated for only a short time a few hours to a few days (see however e.g. *McFenna & Stevens* 1960 *Fuji & Prince* 1964). The highly sensitive methods needed for demonstrating antibodies may not only increase sources of error (cf *Stavitsky* 1961) but may also increase the difficulty in distinguishing between antibody synthesized *de novo* in the culture and antibody synthesized *in vivo* and released during cultivation (cf *Stavitsky* 1963). Only rarely has enough antibody production been obtained *in vitro* to permit its demonstration with immunoprecipitation a method by which some of the difficulties mentioned above could be avoided. *Patterson et al* (1964) utilized the fact that the spleen in humans can

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produce large amounts of precipitating antibodies. In some of their experiments an antibody production *in vitro* could be found of enough strength to permit immunoprecipitation.

The aim of the present investigation was to find a simple method for cultivation of lymphoid cells in suspension for many days and under conditions that made antibody synthesis *in vitro* demonstrable with the use of precipitation methods. The culture method should be simple and easy to modify for various experimental purposes. The source of cells was rabbit lymphoid cells stimulated *in vivo*. The presence of antibody containing cells was studied with immunofluorescence and compared with the presence of antibody in the culture media.

MATERIAL AND METHODS

Antigen. Commercial human γ C globulin (AB Kabi Stockholm) was used as antigen for immunization. A purer preparation (Kabi 1343) was used for immunofluorescence and immunodiffusion studies. (For further details see Nilsson 1967.)

Preparation of lymphoid cell suspensions. A regional lymph node and/or the spleen was teased apart with two forceps in Parke's 199 solution, filtered through double gauze and washed by centrifugation three times with Parker 199. The number of mononuclear cells was determined and suspensions with cell concentrations between 10 and 120 mill/ml were prepared.

Experimental groups. Adult rabbits, weight 2-3 kg, mixed sex, were used. Two groups were studied.

1) **Adjuvant treated controls.** Three rabbits were injected twice intracutaneously, ten days apart, with 0.1 ml 0.9 per cent saline emulsified in the same volume of Freund's complete adjuvant (Disco). Cell suspensions were prepared from regional lymph nodes and spleens four days after the second injection.

2) **Intracutaneously and intraperitoneally immunized rabbits.** Two rabbits were injected with human gamma globulin intracutaneously (20 mg per dose) and intraperitoneally (50 mg per dose) for one month according to the schedule suggested by Boyd (1955). One day and two days respectively after the last intracutaneous injection cell suspensions were prepared from the spleen. On this occasion immunodiffusion revealed antibodies in both rabbits whereas controls revealed antibodies in only one (titer 1:4000).

3) **Intracutaneously immunized rabbits, heterogeneous group (immunization schedule varied).** Seven rabbits were injected intracutaneously with a volume of 0.1 ml of human gamma globulin emulsified with 0.1 ml Freund's complete adjuvant. The antigen dose varied from 1 to 45 mg. Two to five injections were given during 4-4½ months. Cell suspensions were prepared two, three or four days after the last injection. Both immunodiffusion and Coombs test (titers 1:256 and 1:128000) revealed antibodies in serum. Separate cell suspensions were prepared from the regional lymph node and from the spleen in two rabbits. In each of the other five rabbits cells from the lymph node and the spleen were mixed. Thus nine cell preparations were studied from the seven rabbits.

4) **Intracutaneously immunized rabbits, homogeneous group (immunization schedule standardized).** Eight rabbits were given two injections, ten days apart, each injection contained 6 mg human gamma globulin in a volume of 0.1 ml emulsified with 0.1 ml Freund's complete adjuvant. Cell suspensions were prepared from a mixture of regional lymph node and spleen four days after the last injection. Immunodiffusion and Coombs test (titers between 1:128 and 1:512) revealed antibodies in serum.

Culture method. Cells were suspended in a medium containing 70 per cent Parker 199 and 30 per cent antiserum taken 14 days after the first injection. Antibodies were added to the medium 50 IU penicillin and 50 IU streptomycin per ml. This medium is in the following referred to as the culture medium. In contrast to the *deco* medium described below, the culture arrangement. One or more dialysis tubings (8 mm diameter) (Ciba) (Carlisle) (Ciba) (Ciba) are

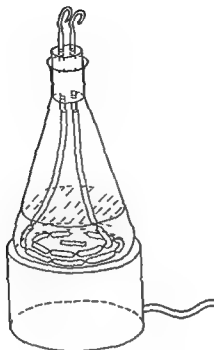


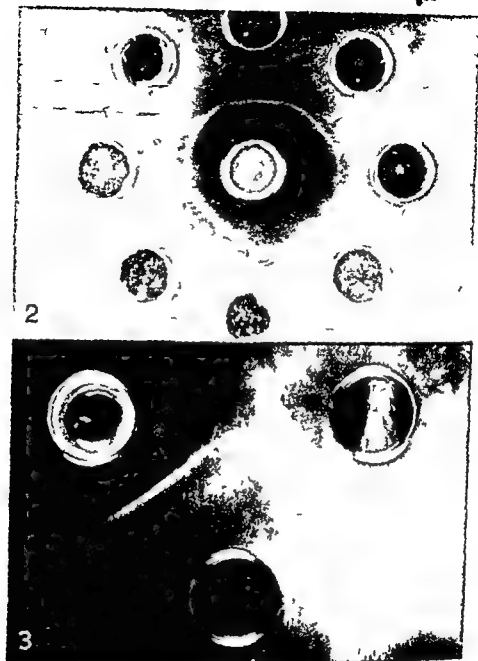
Fig 1

Drawing of the culture arrangement For explanation see text

connected with glass tubes that perforate the stopper of a 500 ml Erlenmeyer flask. The cell suspension is injected into the tubing through a thin rubber tube connected to the external end of the glass tube. In order to distribute the cells along the dialysis tubing it is divided after filling into short segments with perlon thread. The tubing can be divided into separate compartments by tying the tubing itself into knots or separate tubings can be tied together after filling. These steps are performed before the tubing is placed in the flask. The compartments can then be removed separately during cultivation. The flask contains a depot medium (1/3 ml Parker 199 75 ml horse serum and antibiotics in the concentration mentioned) kept in circulation by a magnetic stirrer. In the present study there was no need to renew the depot medium. The flask is placed in a thermostat at 37 °C. Up to three separate dialysis tubings each 90 cm long have been placed in the same flask.

After the end of the culture period the cells were washed three times in saline and used for immunofluorescence studies whereas the culture medium was always concentrated 10–20 times in a collodion bag (Mies 1953) before being analysed for antibodies.

Studies on cell survival during cultivation. Three rabbits were injected intracutaneously with human gamma globulin emulsified with Freund's complete adjuvant according to the schedule of the homogenous group mentioned. Antibodies in sera from these rabbits could be detected by both immunodiffusion and Coombs test (titers between 1:16 and 1:128) four days after the second injection of antigen when cell suspensions were prepared from the regional lymph nodes and the spleen. These rabbits were exclusively used for studies on cell survival. Suspensions with 100 million cells/ml culture medium were cultured as shown schematically in Fig 1 for four, eight and 12 days. At the start of the experiment and after each culture period cells from each preparation were cultured for six hours in test tubes with a cell concentration of 1 million/ml and diluents H₂O (The Radiochemical Centre, Amersham, England, Specific activity 480 mCi/mCi) was added to the culture medium in an amount of 2.5 µCi/ml culture medium. One part of the cell suspension was



Figs 2-3

- Fig 2 Immunodiffusion in agar. On stained slide. In the central well was placed concentrated culture medium. In the peripheral wells were dilutions of the antigen human gamma globulin. In the central well is the antigen human gamma globulin. In the peripheral wells are immune serum and concentrated culture medium respectively. Material from the same rabbit.



Fig 4

Immunoelectrophoresis Unstained slide In the middle well is concentrated culture medium in the side troughs the antigen human gamma globulin Distinct lines at the site of γG

stained with acetic ocean after fixation for a few minutes in 60 per cent acetic acid The total number of cells per ml culture medium and the proportion of cells that in the orcein stained preparations looked healthy were determined at each occasion Another part was used for autoradiography The cells were air dried and fixed with two per cent neutral formal and carefully rinsed Autoradiography was performed with stripping film method using Kodak AR 10 plates and an exposure time of two weeks The cells were stained through the film with hematoxylin

Coombs test Human O Rh+ erythrocytes were coated with anti D serum Uncoated erythrocytes were used as controls Sera were diluted in serial 2 fold steps in saline Culture media concentrates were first diluted 20 times and then further diluted as above

Immunofluorescence method Immunofluorescence was carried out by an indirect method For further details see Nilsson (1967) In the first step human gamma globulin was used preincubated with a pool of normal rabbit serum In the second step fluorescein isothiocyanate (FITC) conjugated goat antiserum globulin vs human serum globulin (Macrobiol Assoc Bethesda Maryland Control 49465) preincubated with the same rabbit serum pool was used Incubation was carried out to eliminate non specific fluorescence (Nilsson 1967)

Two types of control slides were made In type one only normal rabbit serum pool was used in the first step the second step being as above In type two the first step was unchanged but in the second step preincubated FITC-conjugated goat antiserum globulin vs guinea pig serum globulin (Macrobiol Assoc Control 44315) was used

Immunodiffusion in agar gel Serum taken from each rabbit before and after injection schedule and concentrated culture medium from each experiment were studied with Ouchterlony's micro method with a standard equipment (L B Stockholm) 15 μ l of the serum or of concentrated medium was placed in the central well and 5 μ l of the antigen human gamma globulin in the peripheral wells A wide range of antigen dilutions was used Fig 2 shows an analysis of a concentrated culture medium

For verifying the identity between antibody in the culture medium and the serum from each rabbit the following investigation was made An antigen concentration was determined which gave a sharp precipitate both with the concentrated medium and with the immune serum Antigen in this concentration was placed in the central well the concentrated culture medium and the immune serum were placed in the peripheral wells When the precipitates were confluent as shown in Fig 3 it was regarded as a sign of identity between the antibody in the culture medium and that in the serum

Immunoelectrophoresis was performed according to the Eggertson method 10 μ l of serum or concentrated culture medium was placed in the central well After completed electrophoresis 50 μ l of the antigen human gamma globulin was added

in the side troughs. Fig. 4 shows an immunoelectrophoresis of a concentrated culture medium.

Studies on antibody synthesis. The antibody content in the cells of regional lymph node and spleen from rabbits immunized intracutaneously was studied as follows: suspensions with 10^6 mill/ml were prepared in the same way as used for cultivation but the cells were immediately disrupted by freezing and thawing three times. After centrifugation the media were concentrated and analysed with immunodiffusion and Coombs test.

For studies on *de novo* antibody synthesis *in vitro* 0.5 mC of dl valine 1-C^{14} (The Radiochemical Centre, Amersham, England) with a specific activity of 22.9 mC/mM was added to the depot medium in the flask and to the culture medium in the tubings. This gave a ratio between radioactive and non radioactive valine of approx. 1:15. The radioactivity of the media was 1.85 $\mu\text{C}/\text{ml}$. The concentrated culture media were studied with immunodiffusion in the usual way except that 1.3 per cent agar was used (cf. van Furth 1964). The agar slides were washed and dried in the standard way. Stripping film autoradiography using Kodak AR 10 plates was then performed with an exposure time of three weeks.

RESULTS

Studies on Cell Survival during Cultivation

Cell suspensions from three rabbits were cultured with the use of a cell concentration of 60 mill/ml but orcein staining showed that only 43-54 mill/ml looked healthy at the start of the cultivation. After four days in culture 13-24 mill/ml looked healthy; after eight days 5-6 mill/ml; after 12 days 1-3 mill/ml. Autoradiography studies showed strong incorporation of valine H^3 in cells that looked healthy when stained with hematoxylin. A pronounced cell degeneration thus occurs during cultivation; only relatively few cells surviving.

Studies on Antibody Content in Cells and Media after Cultivation

1) **Adjuvant treated controls.** Cell suspensions from three rabbits were divided into 13 parts which were cultured for four or ten days in cell concentrations that varied from 20 to 100 mill/ml of culture medium. The concentrated culture media gave no precipitates with human gamma globulin in immunodiffusion studies and Coombs test was negative with two concentrated culture media studied. The lymphoid cell suspensions, especially after ten days cultivation, also contained large cells that in phase contrast microscopy had the appearance of macrophages. Some of them showed a dark green fluorescence at immunofluorescence microscopy, but this colour differed from the specific apple green fluorescence. This phenomenon was seen also in the two types of control slides (see Material and Methods).

2) **Intravenously and intraperitoneally immunized rabbits and intracutaneously immunized rabbits: heterogeneous group.** These were used in a pilot study for investigating the possibilities of demonstrating antibody in the cells and culture media under various experimental conditions. In all 14 different cell suspensions were cultured from the nine rabbits. Fig. 5 shows the results of immunodiffusion studies.

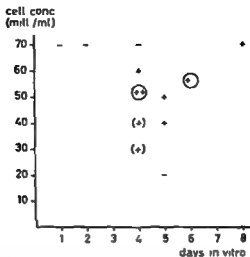


Fig 5

Results of immunodiffusion studies on concentrated culture media. Rabbits belonging to the intravenously and intraperitoneally immunized group and the intracutaneously immunized heterogeneous group. + denotes distinct precipitates permitting reaction of identity (+) denotes faint precipitates not permitting reaction of identity - denotes no precipitates. Two encircled results indicate that two cell preparations (lymph node and spleen) from the same rabbit have been studied separately.

Clear cut precipitates were found in seven experiments. All gave a reaction of identity with the corresponding serum antibody. In a further two experiments weak precipitates were obtained too indistinct to permit a reaction of identity. In three of the seven concentrated media that gave a strong precipitate in immunodiffusion immunoelectrophoresis showed γ G precipitates corresponding to those found in the immune sera of the same rabbits. Coombs test was performed on three concentrated culture media that gave precipitates in immunodiffusion. Two clear cut and one weak and indistinct. The former showed titers of 1:280 and 1:2560 the latter a titer of 1:640. Immunofluorescence studies were made on cells from four cultures with positive immunodiffusion results and from the four negative cultures. Parts from these cell suspensions were also studied with immunofluorescence before the cultivation was begun. Cells with an apple green fluorescence judged specific were found in slides from all parts studied except one taken before the start of the cultivation. In this only very few cells with an apple green fluorescence were seen. This part was therefore scored dubious. After two or more days cultivation of this suspension the investigation gave a clear cut positive result. All control slides (see Material and Methods) were negative. Macrophages with a non specific dark green fluorescence as described above were found in all types of slides.

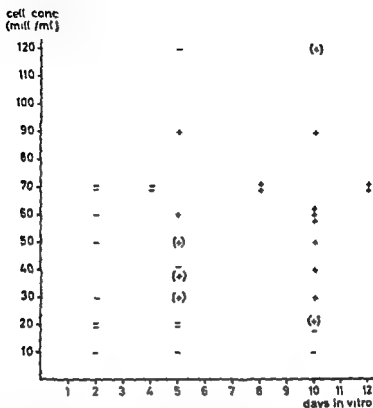


Fig 6

Results of immunodiffusion studies of concentrated culture media. Rabbits belonging to the intracutaneously immunized homogenous group. For symbols see Fig 5.

3) Intracutaneously immunized rabbits homogenous group. This group was used to study the influence of cell concentration and culture duration on the presence in the culture media of antibody demonstrable by immunodiffusion. For this reason other variables were standardized: source of cells, immunization schedule and antigen dose. With cell preparations from these eight rabbits 31 experiments were performed. Fig 6 reports the results. Five days was the shortest culture period that resulted in clear cut precipitates—cell concentrations were 60 and 90 mill/ml, values below or above giving negative or indistinct results. With increasing duration of cultivation positive results were obtained also with lower cell concentrations. With all eight cell preparations positive results were obtained in at least one experiment. All cell suspensions studied were thus able to produce antibody *in vitro*. The negative results obtained can therefore be explained by too short culture periods or unsuitable cell concentrations. Reaction of identity between antibody in immune serum and in concentrated culture medium could be demonstrated with materials from all eight rabbits. Immunoelectrophoresis was performed on positive media con-

centrates from cultures from all these cell preparations and in two γ G precipitates were identified corresponding to those found in the sera from the same rabbits. Seven concentrated culture media from two cell preparations were studied with Coombs test. Medium from one cell preparation was negative after cultivation for two days when studied with both immunodiffusion and Coombs test. After cultivation for five and ten days however the concentrated media showed precipitates in immunodiffusion and Coombs test showed a titer of 1:20. Media from the second cell preparation were negative after two and four days cultivation but after eight and 12 days *in vitro*, precipitates were obtained and Coombs test showed a titer of 1:80. Immunofluorescence studies were made on cell preparations from four rabbits before and after cultivation. In three positive results were found both before and after cultivation. In one cells taken before cultivation were dubiously positive but after two, four and eight days cultivation they were unquestionably positive. After 12 days cultivation the result was again scored dubious because fluorescent cells were few.

Studies on de novo Synthesis of Antibody in vitro

Antibody observed in the culture media may be released from cells that have produced antibody already *in vivo*. The antibody content of washed cell suspensions prepared from the regional lymph nodes and the spleen was therefore studied. Four rabbits that had been injected intracutaneously with human gamma globulin three times during 1-2 months were used. The titers of the sera at Coombs test exceeded 1:4000. The cells from two cell preparations were disrupted in the serum Parker medium. After centrifugation to remove cell debris and after the usual concentration of the medium antibodies were detected by immunodiffusion and these gave a reaction of identity with the corresponding serum antibodies. The concentrated media gave titers of 1:320 and 1:2560 at Coombs test. Cell preparations from the other two rabbits gave after disruption no precipitates but Coombs titers of 1:320. Immunofluorescence was positive for all four cell preparations.

Another three rabbits immunized as the homogenous group gave serum titers at Coombs test of 1:16, 1:128 and 1:512. Cells disrupted in the serum Parker medium gave after concentration no precipitates and Coombs test was negative at 1:20. Immunofluorescence was positive for all three cell preparations.

Thus washed cell preparations from rabbits with high serum antibody titers contained already *in vivo* considerable amounts of antibody and a release of such antibodies during cultivation is perhaps not negligible. Cell preparations from rabbits immunized according to the schedule used for the homogenous group gave no release detectable by the antibody assay methods used. Such cell preparations were there



Fig 7

Autoradiogram of immunodiffusion in agar performed on medium from a culture to which valine C^{14} was added. Concentrated culture medium in the central well and the antigen dilutions in the peripheral wells. Strong specific labelling at the site of precipitates. Weak non specific labelling around central well.

fore studied for further verification that antibody obtained in the culture media was synthesized *in vitro*. A radioactive trace method was used for this purpose.

Cell suspensions from one adjuvant treated control rabbit and from two rabbits belonging to the intracutaneously immunized homogeneous group having Coombs titers of 1:128 and 1:512 were all cultured in media containing valine C^{14} . A culture period of ten days and cell concentrations of 60 mill/ml were used. Both concentrated culture media from the cell preparations taken from the rabbits immunized with human gamma globulin showed precipitates in immunodiffusion. Autoradiography proved that both precipitates contained radioactive material (see Fig 7). The cell suspension from the adjuvant treated control rabbit was divided into two parts: one was cultured with autologous serum in the standard way, the other in a mixture of the immune sera from the two rabbits immunized with human gamma globulin. Precipitating antibodies could as expected be demonstrated only in the medium made up with immune sera. Autoradiography was negative for both parts. Thus no incorporation *in vitro* of radioactive material took place into the antibody synthesized *in vivo* and no trapping of radioactive material occurred at the formation of the precipitates in the control experiment.

DISCUSSION

This paper describes a simple method for cultivation of lymphoid cell suspensions for many days. When cells were taken from rabbits immunized with human gamma globulin antibody was produced in an amount that permitted analysis with immunodiffusion and in some cases with immunoelectrophoresis. Most workers in this field think that a considerably better production of antibody is obtained in organ cultures than in cell suspension cultures. *Stavitsky* (1958), *Askonas & Humphrey* (1958) and others stress the unavoidable trauma that cells are exposed to when a cell suspension is prepared. *Helmreich et al* (1961) quantitized the probable cell damage caused at the preparation of lymphoid cell suspensions and found this to represent a release of not more than five per cent of total cellular aldolase activity. In the present study antibody production has been obtained from cultures prepared by simple teasing of the lymph nodes and spleens. *Pospihil* (1963) suggested that the compact character of lymphatic tissue transferred to tissue cultures permits differentiation processes or promotes cell modulation processes in some other way. *Halliday & Garvey* (1964) and *Bussard & Hannoun* (1966) *inter al* also mention this possibility. *Michaelides & Coons* (1963) point out that the difference can be explained if aeration is important in antibody synthesis *in vitro*. Recently the importance of aerobic conditions has been stressed by *Svehag* (1965) and *Globerson & Auerbach* (1966).

In order to obtain better results with culture of cell suspensions methods have been worked out to increase exchange between cells and gas and fluid phases of the culture. *Steiner & Anker* (1956) placed cell suspensions from immunized animals on a semi permeable membrane in a thin fluid layer to obtain a good exchange with the gas phase above. Below the membrane was put a depot medium that was constantly stirred with a magnetic stirrer. Antibody production was obtained for a few days. The antibody produced *in vitro* was therefore accumulated in a small fluid volume and could be directly demonstrated with immunoprecipitation. This culture method however was judged difficult to handle by for instance *Stevens & McKenna* (1958). The method has recently been used by *Gurvich et al* (1965) and in a modified form by *Neelin* (1965).

Ainis (1962) introduced a still more complicated culture method. This combined Trowell's principle of maintaining the tissues near the surface of the medium as well as certain aspects of Steiner and Anker's chamber. Antibody formation could be demonstrated with microquantitative precipitin test even after as long as 13 days cultivation of lymphoid cell suspensions from immunized animals.

Svehag (1965) cultured lymphoid cell suspensions from immunized animals on membrane filters glued to the top of lucite cylinders each placed in a depression of a plastic tray. Culture medium was placed in

the depressions so that the cell suspensions were situated in the gas fluid interphase. With the sensitive technique of virus neutralization antibodies could be demonstrated in the media after 2-3 weeks cultivation.

In the culture method presented in this paper cell suspensions are not in close contact with the gas phase. Nevertheless, good antibody production has been obtained for many days. Therefore the aeration might not be such a critical feature as previously suggested.

As stressed in the introduction it is necessary to differentiate antibody synthesized *de novo in vitro*, and antibody synthesized *in vivo* and merely released *in vitro*. In early studies the antibody content of media where living cells had been cultivated was compared with that of media where dead cells had been included or with that of extracts of non cultivated cells (cf. Fagraeus 1948). When such a method was used in present context it was found that cell suspensions from rabbits with high serum antibody titers contained considerable amounts of antibody; this might be passively released owing to the pronounced cell degeneration during cultivation. Cell preparations from rabbits with low antibody titers in serum (homogenous group) did not contain enough antibody for demonstration by the used methods. More recently the inhibiting effect of chloramphenicol, actinomycin D or puromycin has been used for indirectly proving the *de novo* synthesis of antibody *in vitro*. Recently Slavitsky & Gusdon (1966) pointed out that actinomycin D can stimulate or inhibit antibody synthesis *in vitro* depending on the concentration used. Slavitsky (1963) has stressed that measurement of the extent of the incorporation of radioactive amino acids into antibody is the only reliable distinction between *de novo* synthesis and release of antibody. Incorporation can be directly demonstrated after precipitation with the homologous antigen. Most authors have then used co precipitation with non radioactive antibody, a sensitive method that might contain large sources of error due to absorption of non specific radioactivity (cf. Slavitsky 1961). Steiner & Linker (1956) obtained amounts of antibody large enough to permit direct precipitation with the antigen and demonstration of radioactivity of the produced antibody. The same goal was reached with the method described in the present paper and indirect methods of demonstration of antibody synthesis could be avoided.

For studying the relative amounts of cells taking part in antibody production immunofluorescence methods can be used. Using this method Patterson *et al.* (1964) found a very high proportion of antibody containing cells—sometimes nearly all the cells in their slides were fluorescent. In the present study relatively few scattered cells contained antibody as demonstrated by this method. No obvious explanation can be given for these divergent results but it has to be pointed out that different experimental systems were used (See also Nilsson 1967). Usually no significant changes in proportions of such cells oc-

curred during cultivation. In only two experiments did a dubious positive result before cultivation become definitely positive after a few days cultivation of the cells. The immunofluorescence results were apparently uninfluenced by cell concentration in the cultures. This contrasts with the above mentioned experiences with antibody analysis of the culture media. Non specific fluorescence in macrophage like cells could slightly complicate the evaluation but it was never a serious problem.

The advantages of the present method for cultivation of lymphoid cells can be summed up in the following points:

1. the culture method is simple and can easily be modified for various experimental purposes.

2. it permits the cultivation for many days of cell suspensions with high cell concentrations.

3. when cells are taken from rabbits immunized *in vivo* antibody production occurs in amounts large enough to permit detection with immunoprecipitation in agar gel and sometimes with immunoelectrophoresis. Direct evidence of synthesis *in vitro* can therefore be obtained from incorporation experiments with radioactive amino acids.

4. the method permits removal of portions of both culture medium and cells at arbitrary intervals during cultivation.

SUMMARY

A simple method for cultivation of rabbit lymphoid cell suspensions for many days is described. Cells taken from rabbits immunized *in vivo* with human gamma globulin produced antibody *in vitro* in amounts large enough to permit analysis with immunoprecipitation in agar. *De novo* synthesis *in vitro* could be directly proved with incorporation of radioactive amino acid into the immunoglobulin in the culture medium.

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THE MONONUCLEAR CELL INFILTRATE IN ALLERGIC CONTACT DERMATITIS

3 Selective Accumulation of Cells from Lymph Nodes

By

STURE LIDÉN

Received 11 II 67

A selective accumulation in allergic contact reactions of lymphoid cells labelled early during the sensitization process has been demonstrated (4-9). These selectively accumulating cells do not constitute any major part of the infiltrating lymphoid cells. It is possible, however, that they represent a hypothetical sensitized cell clone induced to proliferate by the sensitization. The origin of these labelled cells has not been fully clarified. By transfusion of thymic cells Kosunen (6, 7) found but insignificant numbers lodging in reactions of tuberculin type in rats. In an earlier experiment in this series with local thymic labelling the same results were reached in allergic contact dermatitis (10). Locally labelled bone marrow cells on the other hand were found to accumulate selectively in these reactions (11).

Of the peripheral lymphoid organs the lymph nodes have been ascribed a vital role for the elicitation of contact hypersensitivity (2). In the lymph nodes regional to sensitization with contact allergens an increase in the number of large pyroninophilic cells has been found (12-16). The maximal numbers were found on the 4th to 5th days after the sensitizing application. Labelling data indicate that these cells develop into small lymphocytes within the first days (14). It seems reasonable that the lymphocytes thus derived are carriers of immunologic information important for the elicitation of the allergic contact dermatitis. In the above mentioned works Kosunen also transfused cells from lymph nodes regional and non regional to sensitization with tuberculin between inbred rats. After *in vitro* labelling with tritiated leucine he found a selective but non specific accumulation of cells from the regional lymph node compared with the non regional

node (6) After *in vivo* labelling with tritiated thymidine on days 10-12 after sensitization cells collected and transfused on day 14 showed in addition a certain degree of immunological specificity (7)

In a series of experiments on the mononuclear cell infiltrate attempts have been made to achieve what could be called an ideal labelling of different parts of the lymphoid system. Ideal means here that the actual cells are labelled in such a way that they can be traced within the labelled animal itself. In this way the handling of the cells during preparation of cell suspensions and the effects of the transfusion on cells and recipients are avoided. In the present experiment use was made of a method for local labelling of lymph nodes in an attempt to trace cells labelled during the period of maximal large pyroninophilic cell proliferation in the lymph nodes regional to contact sensitization.

The purpose of the work was to compare cells from lymph nodes regional and non regional to contact sensitization with 2,4-dinitrochlorobenzene (DNCB) with respect to their tendency to accumulate selectively in contact allergic reactions. The immunologic specificity of this accumulation was also studied by comparing the percentage of labelled lymphoid cells in reactions to DNCB (used for sensitization of locally labelled lymph nodes) with reactions to 2-phenyl-4-ethoxy-methylene-5-oxazolone (oxazolone) used for sensitization in a non-labelled lymph node station.

MATERIAL AND METHODS

Animals

Twelve white male guinea pigs about 6 weeks old and obtained from the same breeder were used. At the outset the 6 animals labelled in the sensitization area weighed 211 ± 7 g (SD) and the 6 animals labelled contralaterally 198 ± 13 g.

Sensitization

The animals were double sensitized with 2,4-dinitrochlorobenzene (DNCB)¹ and 2-phenyl-4-ethoxymethylene-5-oxazolone (oxazolone). This latter substance was synthesized in the following way according to a method given by Barber & Slack (1). Hippuric acid and ethyl orthoformate were heated with acetic anhydride for one hour under reflux. The low boiling material was removed by a vacuum pump. On cooling the residue solidified. After four recrystallizations from light petrolatum in the cold ($+4^\circ\text{C}$ and -20°C) almost colourless (slightly pinkish) crystals were obtained. Determination of the melting point gave a value of $98.0-100.3^\circ\text{C}$ (compared with $97-98^\circ\text{C}$ according to Barber *et al*).

1 per cent DNCB in acetone (w/v) was applied to the skin of the left axillary region outlined as described earlier (8). 0.2 ml of this solution was distributed over the entire area on day 0 and 1 and 0.3 ml on day 2. This mode of sensitization resulted in but slight necrosis of the treated skin.

The oxazolone was applied as a 10 per cent solution in absolute alcohol of which 0.2 ml was applied to both sides and to the hairless base of one ear on day 0 only. This substance is completely atoxic even at this concentration. However the appearance of the sensitization was observed between days 6 and 8 as an erythema and oedema of the sensitization ear.

¹ Merck AG Darmstadt Germany

Labelling

The labelling was performed with fresh methyl initiated thymidine¹ (H^3 -TdR) with a specific activity of 19 c/mM. The marker was injected intradermally in order to induce local lymph node labelling (8, 13, 15). In a preliminary experiment 8 groups of guinea pigs with 3 animals in each group were given intradermal injections in one of the axillary areas. Each animal was injected twice daily until 7 injections had been given. The animals in the two groups received respectively 1 μ c and 2.5 μ c of H^3 TdR diluted in 100 μ l of 0.9 per cent saline at each labelling occasion. The animals were killed 1 hour after the last injection. The labelling intensity in the lymph nodes regional to labelling after both these doses was fairly high but not optimal for the present experiment even if the maximal individual grain counts exceeded 50. In the control lymph nodes the maximal grain count was 8 in the group which received 2.5 μ c per injection. A few heavily labelled cells present in the control lymph nodes were regarded as migrants from the locally labelled lymph nodes.

Considering the interval of 3 days between the last labelling injection and biopsy in the main experiment the individual labelling doses were increased to 5 μ c of H^3 TdR diluted in 100 μ l of 0.9 per cent saline. The H^3 TdR solutions were never more than 17 hours old when the injections took place. The 5 μ c were injected on 8 different occasions three times a day (injection times 8 a.m., 3 p.m. and 11 p.m.) beginning at 3 p.m. on day 1 and ending at 11 p.m. on day 4. Six animals were labelled in this way in the left axillary area and the area of sensitization and six other animals in the right axillary area. All animals were given a short and superficial ether narcosis (duration about 2 minutes at the most) on every labelling occasion. No side effects were noted from the treatment.

Testing

0.001 per cent DNCB and 0.1% per cent oxazolone dissolved in absolute alcohol were used as test substances. On day 10 one of the test solutions were applied to a circular area measuring 2 cm on the electrically shaved hind parts of the mid flanks. The oxazolone test and one of the DNCB tests was applied 24 hours and the other DNCB test 12 hours before taking the biopsies on day 11.

Skin Biopsies: Histologic and Autoradiographic Technique

Skin biopsies were taken from the three different test sites and from untreated control skin in the vicinity of the 24 hour DNCB test. All biopsies were taken at the same dorsoventral level. They were fixed in formalin and embedded in paraffin. Sections cut at 5 μ from all four biopsies of each animal were placed on the same slide together with a section from a non labelled animal. The positions of the different skin types were changed in order to compensate for possible systematic differences in autoradiographic quality in various parts of the film. For autoradiography Kodak AR 10 stripping film was used. The exposure time was 8 weeks. The slides were stained using haemalum according to Mayer after development of the film.

A more detailed account of the procedure hitherto described has been given in a previous paper (9).

Evaluation of Autoradiogram

The percentage of labelled lymphoid cells in corium and epidermis and the number of grains over the nucleus of the cell was determined. The lymphoid cell were defined according to Groth (3). Macrophages as defined by Kosunen, Blomman, Flax & Fehn (5) were included among the lymphoid cells in corium. The limit for classifying a cell as lymphoid was the same as that employed in the previous investigation in this model. Lymphoid cells in corium showing 3 grains or more and lymphoid cells in epidermis showing 2 grains or more were regarded as labelled. Control of the background was accomplished by counting of 100 lymphoid cells in the sum of the sections on 3 different slides. Of the c

3,200 cells 0.5 per cent had 3 grains or more. This is well below the 2 per cent chosen as upper limit. No differentiation between the different lymphoid cell types was made. In this way 100 lymphoid cells in the epidermis and 1,000 in the corium were counted in sections from each of the four different biopsies taken from every animal. As a rule parts of 8 sections were counted from each biopsy. The maximal grain count number over the skin epithelial cells was determined by screening at least one section from every animal. In an additional counting a further 100 and 1,000 lymphoid cells were counted in the epidermis and in the corium respectively from each of the different biopsies. This time only cells with a grain count exceeding the maximal grain count of the epithelial cells in epidermis were recorded. These cells were considered as probable migrants from the locally labelled lymph node stations.

TABLE 1

The Difference between the Percentage of Labelled Lymphoid Cells in Test Skin (T) and Control Skin (C) in Two Groups of Guinea Pigs Labelled Locally in Different Ways

Treatment	Animal	Per cent labelled lymphoid cells T-C							
		Corium				Epidermis			
		DNCB 1 st hrs	DNCB 24 hrs	Oxa 24 hrs	Control	DNCB 12 hrs	DNCB 24 hrs	Oxa 24 hrs	Control
Labelling of lymph nodes regional to sensitization	1	2.0	1.1	0.7	4.7	—4	5	4	9.0
	2	3.3	4.0	3.9	3.6	13	4	8	13
	3	1.7	0.3	2.0	2.2	3	12	8	10
	4	—0.3	—0.3	—0.8	4.5	15	0	13	11
	5	0.6	0.4	0.8	3.9	5	—1	8	1.7
	6	0.2	0.5	1.1	2.9	0	2	2	13
Mean values		1.3	1.3	1.4	3.6	5.3	3.7	4	13.5
Labelling of lymph nodes contra lateral to sensitization	1	0.6	2.0	0.4	2.3	4	7	0	14
	2	9.0	0.3	—0.3	3.6	8	8		13
	3	5.9	1.9	3.7	2.6	4	4	4	17
	4	1.4	3.3	1.7	2.3	1	1	1	16
	5	—0.9	1.0	0.2	3.7	—4	1	8	2.1
	6	0.1	2.5	0.1	3.6	12	2	1	17
Mean values		1.5	1.9	0.9	3.1	3.2	3.9	2	16.8

In corium 1,000 lymphoid cells and in epidermis 100 lymphoid cells have been counted in each of the four biopsies from every animal.

DNCB = 2,4 dinitrochlorobenzene

Oxa = 2-phenyl-4-ethoxymethylene-5-oxazoline

The values of the control skin are presented in order to indicate the degree of labelling.

RESULTS

The results from the countings recording lymphoid cells with 3 grains and more over their nuclei are presented in Table 1. The values of the test skins have been corrected by subtraction of the corresponding values of the control skin in order to compensate for possible differences in degree of labelling between the animals. Comparison between the animals labelled in the sensitization area and those labelled contra

laterally shows no statistically significant differences for any of the skin types. Nor are there any significant differences between the percentages of labelled cells in the biopsies from the DNCB test sites and from the oxazolone test site. These countings do not even indicate a trend in any direction. The percentages of labelled cells in the test skins however show higher values than in control skin in most instances even if statistical significance is not reached for all the different cell populations counted.

The lymphoid cell responses varied widely between the different reactions but were as a rule high. No correlation was found between the intensity of the reactions measured by this method and the increase in percentage of labelled cells in any of the groups.

The screening of epithelial cells in the epidermis for determination of their maximal grain count values showed a variation between 10 and 15 grains for the different animals. Cells with more than 10 grains were however rare.

TABLE 2
Numbers of Lymphoid Cells with ≥ 16 Grains

		DNCB 12 hrs	DNCB 24 hrs	Oxa 24 hrs	Con trol
Labelling of lymph nodes regional to sensitization	Corium	27 (23)	19 (16)	6 (0.5)	2 (0.2)
	Epidermis	70 (16.7)	4 (3.3)	6 (6.7)	0
Labelling of lymph nodes contralateral to sensitization	Corium	24 (2.0)	21 (1.8)	13 (1.1)	4 (0.3)
	Epidermis	7 (5.8)	2 (1.7)	5 (4.2)	0

From each of the two labelling groups 17 000 lymphoid cells have been counted in corium and 1200 in epidermis from each of the four biopsy types.

Figures in brackets indicate per mille of labelled cells.

The results from the countings of cells showing a grain count exceeding that of the epithelial cells i.e. 16 grains or more are presented in Table 2. From each of the four biopsies 2 000 lymphoid cells in corium and 200 in epidermis were counted. In every type of test reaction and in both animal groups the test skin biopsies show an accumulation of these heavily labelled cells compared with control skin. The proportion of these labelled cells is larger in the corium of the DNCB tests than in the oxazolone tests. This applies to both labelling groups however even if the difference is less pronounced in the group labelled contralaterally to sensitization. The proportion of heavily labelled cells is generally higher in epidermis than in corium. The most obvious difference between the proportion of these cells exists in epidermis of the 12 hour and the 24 hour DNCB tests.

General microscopy of the sections yielded additional data. The majority of the labelled cells were typical lymphocytes (small, medium and large). Labelled cell which undoubtedly were macrophages were

observed but with a very low frequency. Macrophages with a grain count exceeding 15 were occasionally encountered.

DISCUSSION

In a previous investigation the radioactivity in the locally labelled lymph nodes in non sensitized animals was found to decrease successively during the first week at the end of which the level of the control nodes was approximated (8). Thus most of the cells labelled on days 2-4 probably have left the locally labelled lymph nodes at the application of the tests on day 10. As shown by Macher (12) the lymph nodes regional to contact sensitization with DNCB increase in weight during the sensitization. This increase begins at about the 11th day. The H^3 TdR injections given on days 2-4 thus may give labelling of more cells in the animals injected in their sensitization area than in the control group injected contralaterally to this. This fact emphasizes the importance of using the relationship between the autoradiographic values within the same labelling group when comparing the two groups with each other.

The number of lymphoid cells infiltrating the contact allergic reactions varies with the intensity of the reactions. Furthermore the cellular density of the infiltrate varies in different parts of the same biopsy, the lymphoid cell number being highest around vessels, as is the case in delayed hypersensitivity reactions in general (14). Because of these circumstances systematical counting of a certain number of cells probably gives a more representative picture of the percentage of labelled cells than does counting of the number of labelled cells per view field (6-7).

The increase above the control skin level in the percentage of labelled lymphoid cells in all tests but one shown in Table 1 is in agreement with results obtained in experiments using general labelling early during the sensitization process (4-9). It is possible that it is in the present experiment this is an effect of the general labelling resulting from the intradermal injections. If this is the case it may disguise an immunologically specific accumulation of lymphoid cells.

The tracing of heavily labelled lymphoid cells, i.e. cells with a grain count which is larger than the maximal grain count in the epithelial cells of the epidermis gives a somewhat different picture. These countings show that these cells are present in the test skin more frequently than in control skin but also that they have a preference for epidermis compared with corium. With regard to the immunologic specificity no firm conclusions can be drawn even if a tendency to larger differences between the DNCB tests and the oxazolone tests can be observed in the regionally labelled group than in the contralaterally labelled group. The most conspicuous finding is the pronounced difference between the numbers of heavily labelled cells in epidermis of

the 12 hour and the 24 hour DNCB tests in the regionally labelled group. This result tallies well with the concepts of cytotoxicity reviewed by Waksman (17) i.e. that sensitized cells are destroyed on contact with the specific antigen. It might be questioned if all cells with 16 grains or more really are derived from the locally labelled lymph nodes. It is possible that some cells labelled by the systemic labelling effects of the local labelling attempts can reach this degree of labelling. On the other hand most of the heavily labelled cells have 20 to >50 grains numbers which are most unlikely to result from general labelling.

In the present investigation a considerable proportion of the labelled lymphoid cells were small - medium lymphocytes. Only occasionally heavily labelled macrophages were noted. The pattern of labelling is thus different from that observed in a previous experiment with labelling about 3 days before taking the biopsies 48 hours after testing, where the labelled population was dominated by large lymphocytes and macrophages (11). If this difference is caused by the varying labelling techniques, by the times chosen for labelling, or by the different ages of the test reactions is not possible to determine on the basis of available data.

The conclusions of this experiment are based mainly on the results obtained from the countings of the heavily labelled lymphoid cells which are presumed to be largely migrants from the locally labelled lymph nodes. These cells make up part of the mononuclear cell infiltrate in allergic contact dermatitis. They are found in a larger percentage in epidermis than in corium. They constitute a higher percentage of the lymphoid cells in test skin than in control skin i.e. they selectively accumulate in the test skin. No clearcut evidence for an immunologic specificity of this selective accumulation has been found. The findings suggest however that there may be an early accumulation of immunologically specific cells at least in the epidermis and that these cells disappear later possibly as a result of a cytotoxic action of the antigen on those cells. The general labelling pattern showed that lymphoid cells of all sizes were labelled.

SUMMARY

Albino guinea pigs were double sensitized with two different contact antigens DNCB and oxazolone. At the time of the most pronounced proliferation of large pyroninophilic cells in the lymph nodes regional to the sensitization the animals were labelled with tritiated thymidine. In one group of animals a purely local labelling of the lymph nodes regional to sensitization with DNCB was performed. In another group such local labelling was given to the lymph nodes contralateral to those regional to sensitization. A local labelling was given to the lymph nodes contralateral to sensitization with oxazolone. Tests were ap-

plied with both antigens and biopsies were taken after 12-24 hours. Autoradiographical analyses showed that lymph node cells participate in the mononuclear cell infiltrate of the allergic contact reactions. The lymph node cells were more frequent in test skin than in control skin indicating a selective accumulation at the test sites. An immunological specificity of this accumulation could not be established. The lymph node cells showed a preference for epidermis compared with corium. The lymphoid cell infiltrate contained more labelled cells of small-medium size than the infiltrate of tests in a previous experiment where the animals were labelled shortly before testing.

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EXPORT AND IMPORT OF LYMPHOCYTES IN THE THYMUS DURING STEROID INDUCED INVOLUTION AND REGENERATION

By

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Certain steroids particularly the glucocorticosteroids cause characteristic involution of the thymo lymphatic tissue (*Selye* 1936 *Ingle* 1940 *Dougherty & White* 1945) This involution being part of the stress syndrome (*Selye* 1951) is most pronounced in the thymus The mechanism responsible for this thymic atrophy has been studied by many authors with divergent results in certain respects Histologically nuclear pyknosis and lymphocytolysis are reported in the thymic cortex *in vivo* as well as *in vitro* during the first hours after administration of corticosteroids (*Dougherty & White* 1943 1944 1945 *Schrek* 1949 *Feldman* 1950 *Baker et al* 1951 *Ehrlich & Seifter* 1953) The corticosteroids are also stated to inhibit the synthesis of protein and nucleoproteins especially DNA and to decrease the mitotic activity of the thymo lymphatic tissue (*Dougherty & White* 1945 *Studer* 1950 *Engel* 1951 *Hull & White* 1952 *Pfeiffer et al* 1952 *Kass et al* 1953 *Alt & Barron* 1953 *Robbins* 1955 *Clark & Stoerk* 1956 *Blecher & White* 1958 *Lundin* 1958 *Ishidate & Metcalf* 1963 *Bellamy et al* 1966 *Anulson & Lundin* 1966)

In the blood a deficiency of lymphocytes is reported in steroid treated animals (refs *Dougherty* 1959) This may be due to intravascular lysis of lymphocytes and/or to reduced production and delivery of lymphocytes from the thymo lymphatic system In the guinea pig the thymus is a major contributor to the circulating small lymphocytes (*Ernstrom & Larsson* 1966a) and in this animal the export of lymphocytes from the thymus under different conditions can be evaluated by a direct comparison between the lymphocyte content of the arterial blood and that of the thymic venous blood In the present study the migration of lymphocytes to and from the thymus was investigated during steroid induced involution and regeneration of the thymo-lym

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phatic tissue. Furthermore the experiments were extended to include the effect of a corticosteroid on the circulating lymphocyte populations of thymectomized guinea pigs.

MATERIAL AND METHODS

Totally 314 male guinea pigs with an initial weight of 237 ± 9 g (mean \pm S.E.) were used. The animals were fed on cabbage, turnips, carrots and vitamin pellets. They were divided into four main groups (number of animals in brackets):

- Non operated controls injected with saline (43)
- Non operated animals injected with prednisolone (170)
- Thymectomized controls injected with saline (75)
- Thymectomized animals injected with prednisolone (76)

Thymectomy was performed under local anaesthesia (subcutaneous infiltration of 0.1 per cent Xylocain® Astra) according to the technique of *Cyllensten* (1953).

Prednisolone (Ultracortenol® Ciba) was administered i.p. in a dose of 80 mg/kg b.w. In the thymectomized guinea pigs the injection was given immediately after operation. Examinations were made 1, 3 and 6 hours and 1, 3, 6 and 9 days after treatment about 15 animals at each time. The investigations were made between 9 a.m. and 4 p.m. The animals were anaesthetized with 2.5 per cent Nembutal sodium (75–50 mg/kg b.w. i.p.).

In the non operated animals the thymus was exposed and the largest of the thymic veins ligated and cauterized. A sample of blood was taken in a heparinized pipette (Heparin® Vitrum 5000 IU/ml) for supravital staining of white blood cells for white cell counts and for preparation of blood smears.

The right carotid artery was incised near the origin of the thymic artery and samples of arterial blood were taken for analysis as above. Immediately afterwards the artery was ligated. The confluence of the thoracic duct and the left subclavian and jugular veins was then exposed essentially according to the technique of *Reinhardt & Yoffey* (1957). The thoracic duct was punctured and a sample of lymph was collected in a pipette for supravital staining. The whole procedure was performed under a dissection microscope.

The supravital specimens of blood and lymph were stained with Janus green B and neutral red (see *Ernstström & Larsson* 1966b) and were immediately examined in a light microscope at 1000 \times magnification. Differential counts were made of lymphocytes with different mitochondrial content, 100 cells per specimen being counted. The lymphocytes were registered in six classes: cells with 0–5, 6–10, 11–15, 16–20, 21–30 and > 30 mitochondria. For convenience 0, 10, 11, 20 and > 20 mitochondria per cell are denoted as low, medium and high mitochondrial content (ML) respectively.

For determination of the absolute number of white blood cells, 25 mm³ of blood was diluted with 475 mm³ of Toisson's solution (containing methyl violet for staining of white cells). The number of cells was counted in a Bürker counting chamber, 288 squares (corresponding to 0.00625 mm³ each) being counted for each sample.

The blood smears were fixed in methanol for 2 minutes and stained with Giemsa for 12 minutes. They were examined in a light microscope and differential counts were made of lymphocytes, monocytes and granulocytes.

The results were analyzed statistically by Student's *t* test. The comparison between the percentage and number of lymphocytes in thymic vein and carotid artery blood was performed by statistical analysis of all the differences in the individual animals. The *p* values < 0.001 , < 0.01 and < 0.05 are denoted as highly significant, significant and almost significant respectively.

RESULTS

No mortality occurred. The increase in body weight was about 2 g a day irrespective of treatment or operation.

Control Animals

In the controls the lymphocyte content per mm^3 of blood from the thymic vein exceeded that from the carotid artery ($p < 0.001$) again proving the existence of a net emigration of thymic lymphocytes in normal young guinea pigs (see *Ernstrom et al* 1963). A subdivision of the lymphocyte population based on the mitochondrial content of the cells resulted in the following, and no arteriovenous difference in number of cells per mm^3 of blood passing through the thymus the statistical analysis being based on the differences in the individual animals (mean \pm S.E.). Lymphocytes with low mitochondrial content 629 ± 167 ($p < 0.01$). Lymphocytes with medium mitochondrial content 122 ± 67 . Lymphocytes with high mitochondrial content $—8 \pm 9$.

As the mitochondrial content is correlated to the size of the lymphocytes (Wiseman 1931 Fichtelius & Larsson 1961 *Ernstrom & Larsson* 1963) this once more proves that the emigrating thymic cells are small lymphocytes and that no net emigration of large lymphocytes occurs (see *Ernstrom & Larsson* 1966a).

A higher number of granulocytes per mm^3 of blood was noted in the carotid artery than in the thymic vein ($p < 0.001$) indicating an immigration of granulocytes into the thymus. This was not found to be statistically significant in earlier experiments (*Ernstrom & Larsson* 1966a).

In the thoracic duct lymph the medium sized lymphocytes (with medium MC) dominated (65.1 ± 0.8 per cent).

TABLE 1

Percentage and Number of White Cells in Carotid Artery Blood of Control and Steroid Treated Guinea Pig Mean \pm S.E.

Interval after steroid injection	No of animals	Lymphocytes		Monocytes		Granulocytes	
		%	No./ mm^3	%	No./ mm^3	%	No./ mm^3
Controls	43	64.0 ± 1.9	1710 ± 133	3.9 ± 0.9	93 ± 12	32.8 ± 1.8	932 ± 104
1 hour	19	69.3 ± 3.3	1713 ± 213	2.6 ± 0.3	75 ± 15	38.2 ± 3.3	1121 ± 164
3 hours	19	33.8 ± 3.5	1710 ± 23	9.3 ± 0.4	110 ± 16	61.0 ± 3.5	3961 ± 411
6 hours	19	37.6 ± 4.5	1468 ± 153	2.7 ± 0.3	131 ± 23	59.6 ± 4.5	3157 ± 594
1 day	18	67.0 ± 3.2	1933 ± 222	2.5 ± 0.2	79 ± 18	33.5 ± 3.2	1140 ± 172
3 days	15	66.2 ± 2.4	1598 ± 145	2.7 ± 0.3	54 ± 8	31.6 ± 2.4	791 ± 103
6 days	15	63.5 ± 4.0	1916 ± 194	9.9 ± 0.3	91 ± 15	33.6 ± 4.0	1047 ± 157
9 days	15	69.9 ± 2.1	2100 ± 207	9.0 ± 0.2	61 ± 10	28.1 ± 2.0	870 ± 123

Prednisolone Treated Animals

No general lymphocytopenia occurred (Table 1). However subdivision of the lymphocyte population into cells with low, medium and high mitochondrial content (MC)—corresponding to small, medium sized and large lymphocytes—revealed a decreased number of small

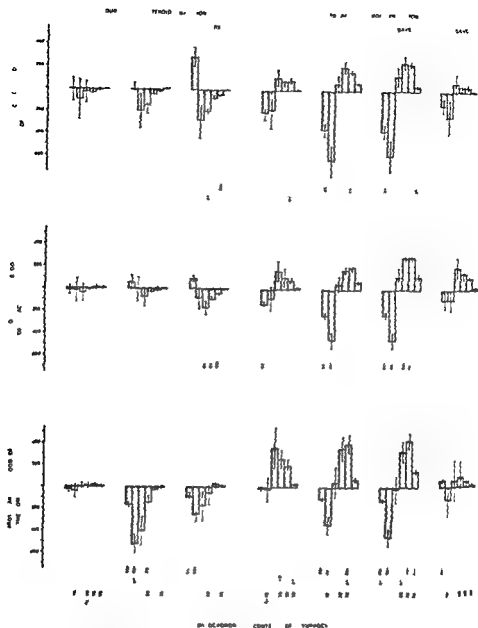


Fig 1

Steroid induced changes in number of lymphocytes with different mitochondrial content per mm³ of blood in the thymic vein and in the carotid artery of guinea pigs with an intact thymus and in the carotid artery of thymectomized guinea pigs. The bars indicate the mean difference (\pm SE) between the number of lymphocytes of different categories in the steroid treated non operated and thymectomized animals on the one hand and that in the normal control and the steroid treated thymectomized animals at the corresponding interval after operation on the other hand.

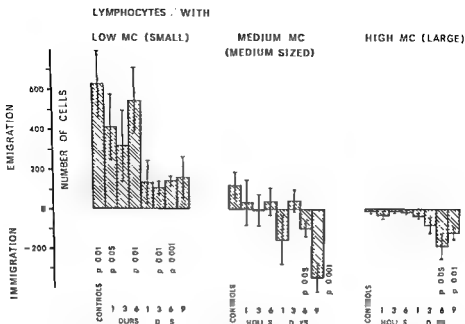


Fig 2

Thymic emigration and immigration of lymphocytes in guinea pigs normally and during steroid induced thymic involution and regeneration (1 3 6 hrs 1 3 6 and 9 days after injection of a thymolytic steroid) determined as the difference between the number of lymphocytes in the thymic vein and that in the carotid artery. The lymphocytes are subdivided into cell with low medium and high mitochondrial content (MC). Mean \pm SE.

lymphocytes and an increased number of larger cells per mm^3 of blood during the late phase (1 3 and 6 days) after steroid injection (Fig 1). The most pronounced change was seen among the smallest lymphocytes with 0-5 mitochondria per cell which were decreased to 15 per cent of the control value 8 days after steroid administration. At 9 days no significant deficiency of small lymphocytes was found whereas an increased number of larger lymphocytes persisted (Fig 1).

The thymic veno-arterial difference in number of lymphocytes with low MC (small cells) was decreased in the steroid injected animals in comparison to that in the controls (Fig 2). This decrease in number of small lymphocytes emigrating from the thymus was significant during the late phase (1 3 6 and 9 days) after prednisolone injection (Table 2). In contrast to normal guinea pigs the animals investigated 6 and 9 days after steroid injection had a larger number of lymphocytes with medium ($p < 0.05$ and $p < 0.001$) and high MC ($p < 0.05$ and $p < 0.01$) in blood from the carotid artery than in blood from the thymic vein (Fig 2). This indicated a net immigration of medium sized and large lymphocytes into the thymus during the regenerative phase after steroid induced thymic involution (for histological details of predni-

solone induced involution and regeneration of the thymus in the guinea pig see Gyllenstein 1962 Ernstrom & Gyllenstein 1965)

At 3 and 8 hours after steroid injection pronounced granulocytosis appeared the number of granulocytes being 350 and 338 per cent of the control value (Table 1) In all the steroid treated animals an excess of granulocytes was found in carotid artery blood in comparison to thymic vein blood

TABLE 2

Thymic Emigration (Veno Arterious Difference in % of Cells/mm³ of Blood) of Small Lymphocytes (characterized by Low Mitochondrial Content in Controls and Steroid Treated Guinea Pigs A Decreased Output of Small Lymphocytes is Demonstrated 1 3 6 and 9 Days after Steroid Injection Mean Difference \pm SE

	Interval after steroid injection							
	Controls	1 hour	3 hours	6 hours	1 day	3 days	6 days	9 days
No of emigrating small lymphocytes	629 \pm 167	413 \pm 165	370 \pm 181	548 \pm 164	175 \pm 109	111 \pm 36	139 \pm 73	156 \pm 106
t	3.77	2.46	1.77	3.34	1.24	3.09	6.04	1.47
df	15	9	11	11	9	10	10	10
p	<0.01	<0.05		<0.01		<0.05	<0.001	
Steroid induced decrease in no of emigrating small lymphocytes		216 \pm 77	309 \pm 246	81 \pm 734	494 \pm 199	518 \pm 171	490 \pm 160	473 \pm 194
t		0.91	1.78	0.35	2.48	3.03	2.90	2.39
df		9	26	76	24	25	25	25
p					<0.05	<0.01	<0.01	<0.05

At 6 hours after steroid injection the percentage of small thoracic duct lymphocytes (with low MC) was increased ($p<0.001$) and that of medium sized and large lymphocytes (with medium and high MC) correspondingly decreased ($p<0.001$ and $p<0.001$) At 3 and 8 days the percentage of small lymphocytes in the lymph was decreased ($p<0.001$ and $p<0.001$) and that of large lymphocytes markedly increased ($p<0.001$ and $p<0.001$) (Fig. 3)

Thymectomized Animals

The total number of blood lymphocytes was decreased 1 hour after operation ($p<0.001$) the decrease comprising all subgroups of lympho-

Fig 3

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph of intact guinea pigs treated with a thymolytic steroid and 8 thymectomized guinea pigs treated with saline or steroid at different intervals after steroid injection and thymectomy Mean \pm SE The arrow indicates injection of steroid and thymectomy

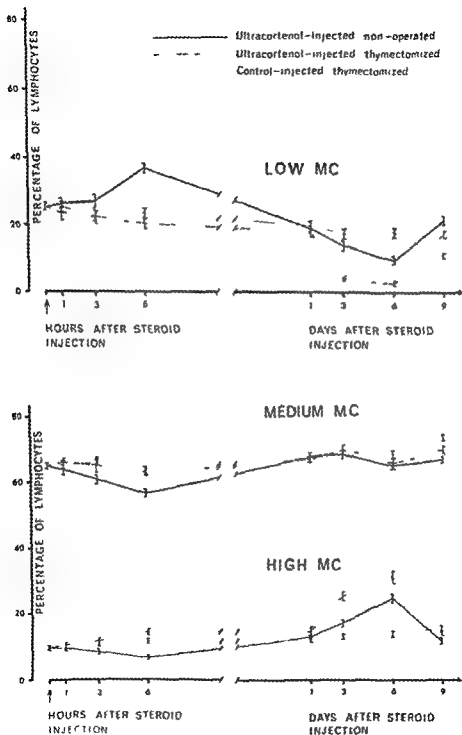


Fig 3

solone induced involution and regeneration of the thymus in the guinea pig see Gyllenstein 1962 Ernstrom & Gyllenstein 1965)

At 3 and 6 hours after steroid injection pronounced granulocytosis appeared the number of granulocytes being 350 and 338 per cent of the control value (Table 1) In all the steroid treated animals an excess of granulocytes was found in carotid artery blood in comparison to thymic vein blood

TABLE 2

Thymic Emigration (Veno Arterious Difference in No of Cells/mm³ of Blood) of Small Lymphocytes Characterized by Low Mitochondrial Content in Controls and Steroid Treated Guinea Pigs 4 Decreased Output of Small Lymphocytes Demonstrated 1 3 6 and 9 Days after Steroid Injection Mean Difference \pm S.F.

	Controls	Interval after steroid injection						
	1 hour	3 hours	6 hours	1 day	3 days	6 days	9 days	
No of emigrating small lymphocytes	679±167	413±168	370±181	48±164	135±109	111±36	139±93	156±106
t	3.77	2.46	1.77	3.34	1.94	3.03	6.04	1.47
df	15	9	11	11	9	10	10	10
p	<0.01	<0.05		<0.01		<0.05	<0.001	
Steroid induced decrease in no of emigrating small lymphocytes		216±237	309±246	81±234	494±199	518±171	490±169	473±198
t		0.91	1.96	0.35	2.48	3.03	2.90	2.39
df		24	26	26	24	20	25	25
p					<0.05	<0.01	<0.01	<0.05

At 6 hours after steroid injection the percentage of small thoracic duct lymphocytes (with low MC) was increased ($p<0.001$) and that of medium sized and large lymphocytes (with medium and high MC) correspondingly decreased ($p<0.001$ and $p<0.001$). At 3 and 6 days the percentage of small lymphocytes in the lymph was decreased ($p<0.001$ and $p<0.001$) and that of large lymphocytes markedly increased ($p<0.001$ and $p<0.001$) (Fig. 3)

Thymectomized Animals

The total number of blood lymphocytes was decreased 1 hour after operation ($p<0.001$) the decrease comprising all subgroups of lympho-

Fig. 3

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph of intact guinea pigs treated with a thymolytic steroid and of thymectomized guinea pigs treated with saline or steroid at different intervals after steroid injection and thymectomy Mean \pm S.F. The arrow indicates injection of steroid and thymectomy

In the lymph of the thoracic duct thymectomy caused a decrease in the percentage of lymphocytes with low MC. A minimum value was recorded 9 days after operation ($p < 0.001$). The percentages of lymphocytes with medium and high MC were correspondingly increased (Fig 3).

Prednisolone Treated Thymectomized Animals

The total number of blood lymphocytes was reduced 3 hours after steroid injection in comparison to both non operated controls and thymectomized animals not given the steroid ($p < 0.001$). Later after injection the total number did not differ significantly from the values in the control treated thymectomized animals at the corresponding time after operation (Table 3). The subpopulations of the blood lymphocytes changed markedly. Thus the steroid caused an extremely pronounced deficiency of small blood lymphocytes in the thymectomized animals in comparison to the corresponding thymectomized controls (Fig 1). A highly significant reduction was noted as early as 3 and 6 hours after steroid injection. At 1 day steroid treatment had not further potentiated the deficiency of small blood lymphocytes present after thymectomy without treatment. At 3 and 6 days after thymectomy and steroid treatment however the steroid had produced a still more accentuated deficiency of small blood lymphocytes than that in the corresponding control treated thymectomized guinea pigs. Then the smallest lymphocytes containing 0-5 mitochondria had practically disappeared from the arterial blood where they normally comprise 16 per cent of the lymphocytes. At 9 days the small blood lymphocytes had reappeared. At 1 day after steroid injection as well as later an increased number of medium sized and large blood lymphocytes was recorded in the steroid treated thymectomized animals in comparison to the corresponding thymectomized controls (Fig 1). This steroid effect was similar to that in the non operated guinea pigs.

The granulocytosis observed 3 and 6 hours after thymectomy was not further potentiated by steroid treatment.

The prednisolone induced increase in percentage of small thoracic duct lymphocytes occurring after 3 hours in the non operated guinea pigs did not take place in the thymectomized animals (Fig 3). The decrease in incidence of small lymphocytes (with low MC) 3 and 6 days after injection of prednisolone into the non operated animals also occurred in the thymectomized animals ($p < 0.001$ and $p < 0.001$ when compared to the corresponding thymectomized controls) and simultaneously the percentages of lymphocytes with high MC were increased ($p < 0.001$ and $p < 0.001$). The incidence of small lymphocytes in the lymph was 25.5 per cent in the non operated controls and at 6 days after treatment 9.4 per cent in the non operated prednisolone treated animals, 17.5 per cent in the thymectomized controls and 2.0 per cent in the thymectomized prednisolone treated animals. Thus

this effect of prednisolone was similar in non operated and thymectomized animals. At this time the combined effect of thymectomy and prednisolone caused almost total depletion of the small lymphocytes in the lymph (Fig 3)

DISCUSSION

In a great number of normal guinea pigs the export of small lymphocytes from the thymus was found to be 629 ± 167 cells per mm^3 of blood passing through the organ. The total blood flow through both thymus lobes in the young adult guinea pig is about 80 mm^3 per minute (Larsson 1966a). This denotes a venous output of small lymphocytes from the thymus of 51×10^3 cells per minute or 74×10^6 cells per 24 hours. The number of small lymphocytes per mm^3 of arterial blood was 991 ± 88 . The blood volume can be estimated at 20 cm^3 (cf Ancill 1956) representing 20×10^6 circulating small blood lymphocytes. Thus the output of small lymphocytes from the thymus is sufficient to exchange the circulating small blood lymphocytes 3.7 times a day.

A single injection of a thymolytic steroid (prednisolone) in the guinea pig reduced the export of small lymphocytes from the thymus to about 20 per cent of the normal value 1, 3, 6 and 9 days afterwards. At the same time the number of circulating small lymphocytes was reduced. It seems more probable that this reduction was caused by the decreased output of lymphocytes from the thymus than by intravascular lymphocytolysis, as the latter effect of a steroid appears within 2 hours of its administration (Dougherty 1959). Autoradiographic analysis has demonstrated a delay of 1-3 days between the synthesis of DNA in the thymic lymphocytes and their appearance in the thymic vein blood in normal guinea pigs (Larsson 1966b). This delay is the same as the time between injection of the steroid and reduction of the output of lymphocytes from the thymus in the present investigation, thus indicating an inhibition of DNA synthesis and cellular proliferation in the thymus as a possible cause of the decreased emigration of lymphocytes.

During the regenerative phase at 6 and 9 days after injection of the steroid, an import of medium sized and large lymphocytes into the thymus was demonstrated. Simultaneously the number of circulating such cells was increased in blood and lymph. Such an immigration of lymphocytes into the thymus was not demonstrable in normal guinea pigs. Although the origin of the imported lymphocytes is not disclosed by the present investigation, the report of Ford & Micklem (1963) indicates the bone marrow as a possible source of lymphocytes immigrating into an involuted and regenerating thymus.

Thymectomy produced changes in the circulating lymphocyte populations similar to those seen in the guinea pigs with a steroid involuted thymus. Thus a decreased number of circulating small lympho-

cytes was demonstrated 1, 3 and 6 days after operation whereas the large lymphocytes were increased in number. Nine days after operation the number of small blood lymphocytes was almost normal. In the present experiment no investigations were made later than 9 days after thymectomy. It is true that the small lymphocytes were almost normal in number at this time. It must nevertheless be pointed out that effects of thymectomy in newborn and young adult guinea pigs have been observed at later intervals after operation (Ernstström 1965 a, b; Ernstström & Larsson 1966).

Steroid treatment of the thymectomized guinea pigs caused a marked deficiency of small blood lymphocytes demonstrable statistically as early as 3 hours after prednisolone injection and most pronounced 3 and 6 days after it. The absence of such an acute deficiency of small blood lymphocytes in the steroid treated guinea pigs with an intact thymus is probably due to the intact thymic export of small lymphocytes during the first 24 hours after steroid injection. Experiments now in progress have even shown an increased blood flow through the thymus during this early phase, thus perhaps indicating an increased outflow of thymic lymphocytes during the first hours after a steroid injection.

In the thoracic duct lymph as well a drastic deficiency of small lymphocytes occurred in the animals which were both thymectomized and steroid treated. The plausible explanation of this finding is that the small lymphocytes in the thoracic duct lymph originate essentially from the thymus at any rate during involution and regeneration of the lymphatic system after treatment with a steroid. Since the lymphocyte population is subsequently normalized even in the thymectomized animals it is evident that other sources can compensate for the thymic contribution of small lymphocytes to the thoracic duct lymph.

SUMMARY

Intact and thymectomized guinea pigs were treated with a single dose of prednisolone or saline. The lymphocyte populations of carotid artery and thymic vein blood and of thoracic duct lymph were studied at different intervals after treatment. The lymphocytes were divided into subpopulations classified by the cellular mitochondrial content which is correlated to the size of the lymphocyte. Special attention was focused on the difference between the lymphocyte number in the blood from the carotid artery and from the thymic vein indicating a thymic import or export of cells. The following observations were made:

1. In the normal control animals there was a thymic export of 629 ± 167 small lymphocytes per mm^3 of blood passing through the thymus. This export was calculated to be sufficient to exchange the circulating small blood lymphocytes 3.7 times a day. No significant import or

export of medium sized or large lymphocytes was demonstrated in the normal animals

2 At 1 3 6 and 9 days after a single injection of prednisolone the export of small lymphocytes from the thymus was decreased to 20 per cent of the normal value and the number and incidence of circulating small lymphocytes were reduced in the blood and lymph At 1 3 and 6 days after steroid injection the number and incidence of medium sized and large lymphocytes were increased in the blood and lymph and at 6 and 9 days after injection an import of such cells into the thymus was demonstrated

3 Thymectomy caused a decrease in the number and incidence of circulating small lymphocytes in the blood and lymph 1 3 and 6 days after operation Conversely the large lymphocytes were increased in number 3 6 and 9 days after operation

4 Prednisolone treatment of the thymectomized guinea pigs resulted in a deficiency of small blood lymphocytes highly significant as early as 3 hours after injection and most pronounced 3 and 6 days after it The smallest lymphocytes had then disappeared from the blood and lymph At 1 3 and 6 days after injection the medium sized and large lymphocytes were increased in number and incidence in the blood and lymph in the same way as in the non operated animals

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CHANGES IN BLOOD FLOW THROUGH THE THYMUS IN STEROID TREATED GUINEA PIGS WITH CALCULATION OF THYMIC EXPORT AND IMPORT OF LYMPHOCYTES



By

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The thymus of the guinea pig was shown by direct methods to be a potent contributor of lymphocytes to the circulating blood (*Ernstrom Gyllenstein & Larsson 1965*). This normal thymic export of cells via the efferent blood proved to consist mainly of small lymphocytes (*Ernstrom & Larsson 1966*).

The technique for direct measurement of the export of lymphocytes from the thymus offers new possibilities of studying the regulation of lymphocyte production and release. Among the factors known to influence this regulation are thyroid and steroid hormones. The thymic output of small lymphocytes was in fact greatly increased soon after administration of thyroid hormone (*Ernstrom & Larsson 1965*). Glucocorticosteroids cause characteristic involution followed by regeneration of the thymo-lymphatic system (*Selye 1936 Ingle 1940 Dougherty & White 1945*). These changes are most pronounced in the thymus where well defined histological changes as well as inhibited cell formation have been described. The normal thymic export of 629 ± 167 small lymphocytes per mm^3 of blood passing through the thymus in the guinea pig was found to decrease to 20 per cent at 1, 3, 7 and 14 days after a single injection of prednisolone (*Ernstrom & Larsson 1967*).

For estimation of the lymphocyte producing capacity of the thymus in normal and prednisolone treated guinea pigs it is of essential interest to measure the blood flow through the organ. A method for measuring the blood flow through the thymus was recently described (*Larsson 1966*). When the difference between the total number of lymphocytes per mm^3 in thymic vein and carotid artery blood is known as well as the blood flow through the thymus per minute the total output of lymphocytes per minute can be calculated.

MATERIAL AND METHODS

Totally 72 male guinea pigs with an initial body weight of 281 ± 4 g (mean \pm S.E.) were used. 23 of them as untreated controls. The rest received a single intra-peritoneal injection of prednisolone (Ultracortisol® Ciba) in a dose of 10 mg/200 g b.w. The prednisolone treated animals were examined 3 and 6 hours and 1, 3, 6 and 9 days after the injection. They were anaesthetized with sodium Nembutal (0.3 ml/200 g b.w. of a 2.5 per cent solution i.p.). A femoral vein was exposed for administration of an anticoagulant (Heparin® Vitrum 3000 IU i.v. per 200 g b.w.). A 5 cm incision was made in the neck and the right or left internal jugular vein was exposed. The vein was ligated proximally and distally to the confluence of the thymic veins as were all other small veins emptying into the internal jugular vein except the thymic veins (usually 2 or 3 in number). In some of the controls and some of the animals treated with prednisolone and dissected 6 hours afterwards the thymic veins were ligated and instead the veins from the submandibular gland were left open. A heparinized polyethylene catheter (outer diameter 1.2 mm, inner diameter 0.75 mm) could easily be introduced through a minor incision in the wall of the jugular vein and the blood flow through the thymus or the submandibular gland was led to a small container placed on a Mettler precision balance on which direct continuous readings could be made. A constant flow of 20–30 minutes was required. All dissections were made on a heating unit at 35°C. Immediately after dissection the wound was covered with liquid paraffin at 35°C which was changed repeatedly during the operation. The blood container on the Mettler balance was covered to prevent vaporization. The distal end of the catheter was placed so far below the proximal end that the capillary resistance of the catheter and the siphon effect equalized each other.

In most animals the right side was catheterized but the left side was also used. Both sides were catheterized in some of the controls. After dissection the animals were killed.

The results were analyzed statistically by means of Student's *t* test. The *p* values <0.05 , <0.01 and <0.001 are denoted as almost significant, significant and highly significant respectively.

RESULTS

No differences were recorded between the right and the left side.

In the control animals the blood flow through one thymic lobe was determined to be 41.0 ± 4.9 mm³/minute (Table 1). The blood flow through the submandibular gland was 9.5 ± 8.5 mm³/minute (Table 2).

TABLE 1

Changes in the Blood Flow Through the Right or Left Thymic Lobe at Different Intervals After a Single Injection of Prednisolone. Mean \pm S.E.

Time after steroid treatment	No. of determinations	Body weight (g)	Thymic weight (mg)	Blood flow through right or left thymic lobe (mm ³ /min)
Controls	21	286 ± 11	737 ± 13	41.0 ± 4.9
3	9	257 ± 10	191 ± 17	84.3 ± 9.3
6 hours	8	266 ± 8	187 ± 15	97.7 ± 7.1
24	7	280 ± 17	234 ± 18	6.4 ± 7.6
3	6	303 ± 8	206 ± 15	57.5 ± 9.6
6 days	8	309 ± 4	270 ± 22	51.0 ± 6.1
9	6	270 ± 10	207 ± 26	47.1 ± 3.9

In the prednisolone treated animals the blood flow through the thymus was found to be highly significantly raised 3 hours after steroid injection (to $84.9 \pm 9.3 \text{ mm}^3/\text{min}$) and after 6 hours the increase was still greater (to $97.6 \pm 7.1 \text{ mm}^3/\text{min}$). At 24 hours the increase in blood flow was less pronounced (to $62.4 \pm 7.6 \text{ mm}^3/\text{min}$ $p < 0.05$). Later no significant difference was noted in comparison to the controls and the values slowly decreased at 9 days being almost the same as those in the controls (Table 1). The blood flow through the submandibular gland was not found to be significantly changed in the steroid treated animals as compared to the controls (Table 2).

TABLE 2

Changes in the Blood Flow Through the Right or Left Submandibular Gland 6 Hours After a Single Injection of Prednisolone Mean \pm S.E.

Time after steroid treatment	No. of determinations	Body weight (g)	Salivary gland weight (mg)	Blood flow through right or left submandibular gland (mm^3/min)
Controls	5	284 ± 6	208 ± 22	95.7 ± 8.5
6 hours	5	276 ± 5	204 ± 11	85.4 ± 7.0

DISCUSSION

The blood flow through the thymus was increased soon after treatment with prednisolone this increase being maximal 6 hours after steroid administration. At this time no effect was recorded on the blood flow through a non lymphatic organ i.e. the submandibular gland. This indicates that prednisolone may have a selective effect on the blood flow through the thymus (and perhaps other lymphoid organs) during its involution although an effect on the blood flow through other non lymphatic organs than the submandibular gland obviously cannot be ruled out.

At 3 days and later after the steroid injection the blood flow through the thymus was increased but did not differ significantly from that in normal guinea pigs.

Using the earlier found mean values of the cellular export of lymphocytes of different categories from the thymus of normal and steroid treated guinea pigs (Frnstrom & Larsson 1964) and combining them with the present data on the blood flow through the thymus the export of lymphocytes of different categories can be calculated (Table 3). In this way an initially increased output of small lymphocytes from the thymus is disclosed. From 24 hours after steroid injection and onwards the various output of small thymic lymphocytes decreased. No export of large lymphocytes from the thymus was observed during

the experimental period. On the contrary, negative values were calculated. These were most pronounced 6 and 9 days after injection of prednisolone indicating an immigration of large lymphocytes into the thymus during the regenerative period after treatment with steroid.

TABLE 3

Calculation of the Thymic Output per Minute of Small, Medium Sized and Large Lymphocytes at Different Intervals After a Single Injection of Prednisolone

Time after steroid treatment	No. of Lymphocytes		
	Small	Medium sized	Large
Controls	51.6×10^3	10.0×10^4	-0.7×10^3
3	54.3×10^3	-0.8×10^3	-1.4×10^3
6 hours	107.0×10^3	7.8×10^3	-0.5×10^3
24	16.8×10^3	-13.3×10^3	-4.7×10^3
3	12.8×10^3	5.1×10^3	-9.4×10^3
6 days	14.2×10^3	-9.6×10^3	-18.9×10^3
9	14.7×10^3	-32.2×10^3	-11.3×10^3

SUMMARY

The blood flow through the thymus and the submandibular gland was measured in guinea pigs normally and during steroid induced involution and regeneration of the thymic lymphatic system.

Steroid injection had a stimulating effect on the blood flow through the thymus which was maximally raised 6 hours after injection and gradually decreased to normal during the following week. At 6 hours no effect was found on the blood flow through the submandibular gland.

The present data on the blood flow through the thymus of normal and steroid treated guinea pigs were combined with previous results regarding the thymic veno-arterial difference in number of lymphocytes of different categories. An initial increase and later a pronounced decrease in the thymic export of small lymphocytes were revealed. During the regenerative period of the thymus a net import of large lymphocytes into the organ was demonstrated.

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EXPORT AND IMPORT OF ^3H THYMIDINE-LABELLED LYMPHOCYTES IN THE THYMUS OF NORMAL AND STEROID TREATED GUINEA PIGS

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The induction by corticosteroids of involution and regeneration of the thymo lymphatic system is well documented (Selye 1936 Ingle 1940 Dougherty & White 1945). Their mode of action especially that of the glucocorticosteroids on the thymo lymphatic organs is twofold: first a lysis of cells during the first hours after administration (Dougherty & White 1943 1945 Schrek 1949 Feldman 1950) and secondly a decreased cell production occurring later and probably due to an inhibition of DNA synthesis (refs. see Ernstrom & Larsson 1967).

These steroid induced changes are most marked in the thymus. As this organ is a major contributor of the circulating small blood lymphocytes in the guinea pig (cf. Ernstrom Gyllenstein & Larsson 1966) it was of interest to investigate the thymic export during steroid induced involution and regeneration (see Ernstrom & Larsson 1967 Larsson 1967). Further information on the migration of lymphocytes into and from the thymus after steroid treatment has now been gained by autoradiographic studies of newly formed cells both normally (Larsson 1966) and after steroid treatment (the present paper).

The autoradiographic technique has earlier been used to study the transport of cells within the thymo lymphatic tissue (Cronkite et al 1959 Schooley et al 1959 Loffey 1960 Diderholm 1961 Gouans 1963 Cannon & Whissler 1965 Lundmark & Fuchtelius 1965). The application of autoradiography to the blood of afferent and efferent vessels of the thymus for investigation of its cellular kinetics has not however been tested previously.

MATERIAL AND METHODS

Totally 69 young male guinea pigs with an initial weight of 150 g (mean \pm S.E.) were used. 10 of them as controls. (The controls were the same as those in an earlier experiment Larsson 1966). All the animals received a single intraperitoneal injection of tritiated thymidine (TRA 170 Thymidin-methyl T). The Radiochemical Centre Amersham England) in a dose of 0.5 mCi/kg b.w. 2 days before dissection. The steroid treated animals were given a single i.p. injection

of prednisolone (Ultracortenol® Ciba) in a dose of 50 mg/kg bw 1 3 and 6 hours and 1 3 6 and 9 days before dissection. In each group 7 to 10 animals were examined. The animals were anaesthetized with sodium Nembutal (25-50 mg/kg bw ip). The thymus was exposed and a thymic vein incised. Blood samples were collected in heparinized blood pipettes (Heparin® Vitrum) for preparation of blood smears and for white cell counts in a Burkner chamber. The right carotid artery was incised close to the origin of the thymic artery and blood samples were taken for the same analysis as mentioned above. The artery was then ligated. Finally the thoracic duct was exposed at its confluence with the left subclavian and jugular veins mainly according to the technique of Reinhardt & Loffey (1957) although the sternum was not incised. The duct was punctured and lymph collected for preparation of smears.

For counting of white cells in the Burkner chamber 25 mm³ of blood was collected in a pipette and diluted with 475 mm³ of Tolisson's solution containing methyl violet for staining of the white cells. Totally 288 squares were counted for each determination.

All the smears were allowed to dry in air and were then fixed in methanol for 5 minutes. After drying the smears were covered with a thin film of celloidin (0.5 per cent celloidin in an alcohol/ether solution) and dried again. The slides were dipped into the photographic emulsion (Ilford Nuclear Research Emulsion K5 diluted in two parts of re-distilled and filtered water, dark room) and dried for 3 hours at 20°C. Exposure proceeded for 1 month in absolute darkness at +4°C. The slides were developed (Kodak D 19B) for 3 minutes, fixed for 15 minutes and washed in running water for at least 20 minutes. While still wet the slides with the blood and lymph smears were stained in Giemsa solution (200 drops in 100 ml of re-distilled and filtered water) for 60 minutes. Finally the smears were dipped into alcohol of 70, 90, 95 and 100 per cent covered with celloidin and a cover slip put on.

The cell counts were performed in a light microscope at a magnification of 500 to 1000 \times . The background labelling was as a rule scanty. A minimum load of 3 grains/cell was required for the cell to be regarded as labelled. Altogether 100 to 400 cells were counted in each smear. The percentage of labelled and unlabelled lymphocytes, monocytes and granulocytes was registered. The percentage of labelled lymphocytes was calculated from the percentage of total lymphocytes. The absolute numbers of labelled lymphocytes were calculated from the total number of white cells and the percentage of labelled lymphocytes.

The results were analyzed statistically by Student's *t* test. The *p* values <0.05, <0.01 and <0.001 are denoted as almost significant, significant and highly significant respectively. The comparisons between the number of labelled lymphocytes in thymic vein and carotid artery blood were performed by statistical analyses of the differences in the individual animals.

RESULTS

The percentage of labelled lymphocytes in thymic vein and carotid artery blood was raised 3 and 6 hours after steroid injection (Fig. 1), the increase being highly significant in the arterial blood. Thus the normally lower percentage of labelled lymphocytes in carotid artery blood had reached the values of the thymic vein blood after 3 hours. One day after steroid treatment a parallel decrease in the percentage was noted in both thymic vein ($p < 0.01$) and carotid artery blood ($p < 0.05$) compared with the values at 6 hours. The percentage of labelled lymphocytes in the afferent thymic blood had then returned to the normal level whereas that in efferent thymic blood was decreased below it ($p < 0.01$). Three days after steroid administration the percentage of labelled lymphocytes was further decreased in both thymic vein and carotid artery blood, the values being almost the same. At 6

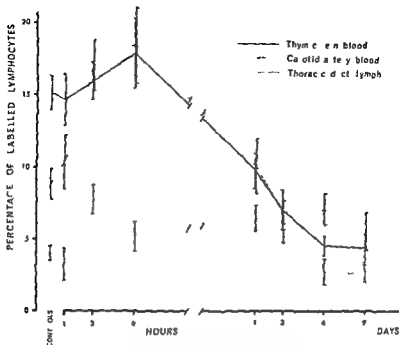


Fig 1

Changes in the percentage of labelled lymphocytes in thymic vein blood carotid artery blood and thoracic duct lymph at different intervals after a single injection of prednisolone Mean \pm SE

and 9 days after injection the percentage of labelled lymphocytes in carotid artery blood exceeded the corresponding values in thymic vein blood although the differences were not significant. At these times the percentages in thymic vein blood were markedly lower than in the controls ($p < 0.001$ and $p < 0.001$ respectively): amounting to only 30 per cent of the normal value.

During the experimental period the absolute number of labelled lymphocytes (Fig 2) showed similar variations to those in the percentage values. This applied to both thymic vein and carotid artery blood. Thus the maximal number of labelled lymphocytes was found 3 hours after injection of steroid, the increase being highly significant in carotid artery blood. The fewest labelled lymphocytes were recorded at 6 and 9 days after steroid treatment. At 6 and 9 days the number in carotid artery blood exceeded that in thymic vein blood, this difference being almost significant on day 6. The number of labelled lymphocytes in thymic vein blood was then only 30 per cent of the normal value.

The veno-arterial difference in labelled lymphocytes per mm³ of blood (Fig 3) was highly significant in the controls and the animals investigated 1 hour after steroid administration, not significant 3 and 6 hours after injection and significant 1 day after. At 3 days no difference was present. Six days after steroid treatment the difference be-

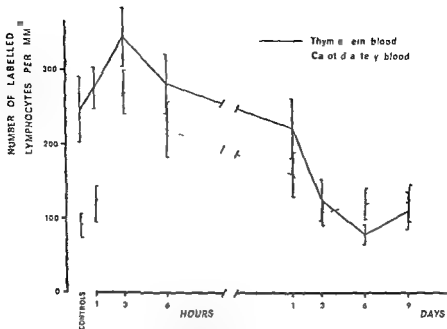


Fig 2

Changes in the number of labelled lymphocytes per mm^3 in thymic vein and carotid artery blood at different intervals after a single injection of prednisolone. Mean \pm S.E.

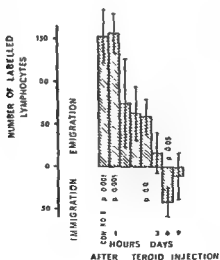


Fig 3

Changes in the thymic veno arterial difference in number of labelled lymphocytes per mm^3 at different intervals after a single injection of prednisolone. Mean \pm S.E.

came negative (i.e., more labelled lymphocytes in carotid artery blood than in thymic vein blood $p < 0.05$) indicating a net immigration of labelled cells into the thymus. At 9 days no veno-arterial difference was observed.

The percentage of labelled lymphocytes in thoracic duct lymph (Fig. 1) was increased 8 hours and 1 day after steroid injection ($p < 0.01$, $p < 0.05$). At 6 and 9 days after steroid treatment the percentage was decreased below the normal level although not significantly.

DISCUSSION

The normal export of small lymphocytes from the thymus was recently determined to be 629 ± 167 cells per mm^3 of blood passing through the organ (Ernstström & Larsson 1967). A single injection of tritiated thymidine does not label more than about 20 per cent of the small lymphocytes in the thymic cortex and in the present study the veno-arterial difference in labelled lymphocytes was 153 ± 31 cells per mm^3 of blood.

The glucocorticosteroids are known to cause on one hand lysis of the already formed cells in the thymic cortex on the other hand inhibition of cell proliferation there probably by interfering in DNA synthesis (for references see Gyllenstein 1962, Ernstström & Gyllenstein 1965). The present study indicates the occurrence of an increased delivery of newly formed lymphocytes to the blood during the first few hours after a single injection of prednisolone (Fig. 2). During this early period after steroid injection the blood flow through the thymus is increased (Larsson 1967). This increased blood flow results in a raised output of lymphocytes from the thymus (Larsson 1967) thus explaining the high incidence of labelled lymphocytes in the blood noted 3 hours after steroid injection. Since 1–3 days elapse between DNA synthesis and migration of the thymic lymphocytes from the organ (Larsson 1966) the decreased production of thymocytes is not reflected until about 3 days after steroid injection. The number of labelled lymphocytes per mm^3 in thymic vein and carotid artery blood was then almost identical possibly due to inhibition of the export of newly formed lymphocytes from the thymus. Later i.e. 6 days after steroid injection there was a negative veno-arterial difference in number of labelled lymphocytes per mm^3 indicating a net import of newly formed cells into the thymus. This is in agreement with recent findings i.e. that 6 and 9 days after a single steroid injection a net immigration of medium sized and large lymphocytes into the thymus occurs (Ernstström & Larsson 1967). These cells may originate in the bone marrow (Ford & Nicklem 1963). Nine days after steroid injection no thymic veno-arterial difference was present and thus no export or import of newly formed lymphocytes in the thymus was established.

In the thoracic duct lymph a significant increase in the percentage

of labelled cells was recorded 3 hours after injection. Although this may have been due to passage of small thymic lymphocytes from the blood to the lymph during the early period after steroid injection the newly formed lymphocytes may also have been of extra thymic origin. Later during involution and regeneration following steroid treatment the percentage of labelled lymph cells did not differ significantly from the control value.

TABLE 1

Calculation of the Thymic Output of Labelled Lymphocytes per Minute at Different Intervals After a Single Injection of Prednisolone

Time after steroid injection	No of animals	Body weight (g)	Thymic export or import of lymphocytes/min
Controls	9	241 \pm 7	6773
3	7	248 \pm 11	6378
6 Hours	6	250 \pm 9	6149
24	10	269 \pm 9	3689
3	10	259 \pm 4	978
6 Days	10	249 \pm 3	-2149
9	8	262 \pm 2	-518

If the present values of veno arterial differences per mm³ of blood are combined with earlier data on the blood flow through the thymus after steroid treatment (Larsson 1967) the total export and/or import of cells in the thymus per minute can be calculated (Table 1). In the controls and soon after steroid administration an output of labelled lymphocytes occurs. Later during regeneration of the thymus a net import of labelled lymphocytes is found. This is in accordance with earlier observations (Ernstrom & Larsson 1967; Larsson 1967).

Theoretically a steroid induced change in the re utilization of thymidine and of the size of the thymidine pool in the thymus may have influenced the appearance of labelled lymphocytes during the latter part of the experiment when ³H thymidine was given after the steroid. A decreased output of lymphocytes from the thymus after steroid administration is however in agreement with earlier findings achieved by other methods (Ernstrom & Larsson 1967).

SUMMARY

Normal and steroid treated guinea pigs were investigated 3 days after an intraperitoneal injection of tritium labelled thymidine. The percentage and total number of labelled lymphocytes in afferent and efferent thymic blood and in thoracic duct lymph were registered. The steroid treated animals were examined at different intervals after steroid injection. The following findings were made:

1 The percentage of labelled lymphocytes in thymic vein and carotid artery blood was increased 3 and 6 hours after steroid treatment. Later the percentage decreased so that 3, 6 and 9 days after injection it was markedly lower than in the controls.

2 The absolute number of labelled lymphocytes per mm^3 in thymic vein and carotid artery blood varied similarly to the corresponding percentages. Thus the number of labelled lymphocytes was greatest 3 hours after steroid injection and smallest during the regenerative period of the thymus.

3 The veno-arterial difference in number of labelled lymphocytes per mm^3 of blood was decreased soon after steroid injection. Six days afterwards during the regenerative phase of the thymus there was a net import of newly formed labelled lymphocytes into the thymus.

4 The percentage of labelled lymphocytes in thoracic duct lymph was increased soon after steroid treatment. During the regenerative period the percentage was decreased.

5 The present values of thymic veno-arterial differences in labelled lymphocytes per mm^3 of blood were combined with earlier data on the blood flow through the thymus after steroid treatment. An export of labelled cells was calculated soon after injection whereas during the regenerative period on the contrary an import occurred.

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1 The percentage of labelled lymphocytes in thymic vein and carotid artery blood was increased 3 and 6 hours after steroid treatment. Later the percentage decreased so that 3, 6 and 11 days after injection it was markedly lower than in the controls.

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but preliminary experiments with retinoic acid indicated that much smaller doses were sufficient to induce teratogenesis. Mother animals were sacrificed on the morning of day III of pregnancy for mice and day 20 for rats. The fetuses were examined for external malformations either fresh or after Poulin's fixation. For visualization of skeletal defects they were cleared in potassium hydroxide and stained with alizarin red.

RESULTS

The data in Table 1 compare the effects produced by retinoic acid and retinyl acetate treatments on the reproductive performance of A/Jax mice. Three dose levels of these compounds could be compared on the basis of obtainability of both a similar range of subsequent embryonic death and resorption and a comparable incidence of fetal malformations.

TABLE 1

Comparison of Effects of Retinoic Acid and Retinyl Acetate on the Reproduction and Malformations in Mice Treated as Given only on a Single Day

Agent	Dose/20gm bodyweight		Days of treatment	Number of animals	Number of implantation sites	Embryos resorbed	% Survivors malformed
	mg	iu					
Retinoic acid	4	1000	8	5	42	100	-
			9	2	16	100	-
			10	4	30	97	100
Retinyl acetate	40	40 000	9	4 (2 died)	18	94	100
Retinoic acid	2	500	9	7	50	70	100
			9	5	37	32	44
Retinyl acetate	20	20 000	8	11	93	75	17
			9	14 (4 died)	80	36	47
Retinoic acid	1	250	8	5	41	74	45
			9	5	37	16	43
Retinyl acetate	10	10 000	8	2	21	29	20
			9	3	26	8	80
			10	4	28	27	33

Only one fetus survived which had severe malformations

Administration of 4 mg of retinoic acid or 1000 iu according to van Dorp & Arcus (1946) on any of 8, 9 or 10 days of gestation was very toxic to the fetuses. All but 1 out of a total of 88 implantation sites resorbed in response to this treatment. The lone survivor was very severely malformed with a shortened trunk and extreme exophthalmos and had several of the defects to be mentioned later for the fetuses treated with milder doses. The dose of retinyl acetate having a comparable lethal effect on the fetuses was in the range of 40 mg (40 000

11) This treatment was toxic for the pregnant animal as well two of the 4 treated mothers died and the remaining animals yielded only 1 living fetus out of 18 implantation sites. This fetus also had all the defects which are described below.

Administration of 2 mg retinoic acid (500 i.u.) on a single day was a very teratogenic dose without having an extreme toxic effect on the fetuses. For some unknown reason two batches of animals treated two months apart first in January 66 and the second in March 66 gave quite different responses. Seven animals in the first batch treated on day 9 yielded 13 living fetuses all very severely malformed. All of these fetuses looked alike and the abnormalities they had could be described as comprising a syndrome. These fetuses had markedly shortened trunk, extreme exophthalmia, microcephalia, stomia rudimentary or missing mandible, tail stump, no genital opening, malformed sternum and ribs, missing vertebrae and some degree of agenesis of the hind limb. Spina bifida occulta in the sacral region occurred in 9 out of 15. The second batch of 5 animals again treated on day 9 yielded 25 living fetuses (a resorption rate of 32 per cent compared to 70 per cent in the first batch) with no typical syndrome as described above. Eleven fetuses among these had abnormalities (44 per cent) such as cleft palate and some rib malformations. The dose level of retinyl acetate which gave a comparable pattern of malformations was again toxic for the mother where 4 out of 14 treated animals died. A dose of 20 000 i.u. of retinyl acetate on day 9 produced 36 per cent resorption. Twenty-four out of 51 live fetuses obtained had various malformations. 8 of these had a typical syndrome described for retinoic acid: all with spina bifida occulta. Fifteen others had cleft palate, these fetuses also had one or more of such malformations as stumpy tail, malformed ribs, hind limb agenesis and exophthalmia. One fetus had only rib abnormalities. 20 000 i.u. retinyl acetate on day 8 produced quite high resorption rate (75 per cent) but a low rate of malformations: only 4 out of 23 fetuses had cleft palate and some rib and sternum malformations. One female treated with 500 i.u. of retinoic acid on day 8 died.

With 1 mg retinoic acid (250 i.u.) although a slightly higher resorption rate (24 per cent) was obtained on day 8 than what it was on day 9 (16 per cent) the per cent incidence of malformations in the fetuses was identical on these two days. Major abnormality encountered was cleft palate: it occurred in 13 out of 14 fetuses on the earlier day and 10 out of 13 on the later. Many of these fetuses showed dermal ribs on the free side. Other abnormalities observed were microphthalmia and anophthalmia. There were no malformations of the type observed with higher doses. The results with this lower dose of retinoic acid compare well with those obtained with 10 mg (10 000 i.u.) of retinyl acetate. Most commonly observed abnormality here was also cleft palate with facial dermal ribs: there were some eye malformations as well as a few skeletal abnormalities in the tail, sternum & ribs.

TABLE 2

Comparison of Effects of Retinoic Acid and Retinyl Acetate on the Reproduction and Malformations in Rat Indicated Dose Was Given Daily for 3 Days

Agent	Dose		Days of treatment	Number of animals	Number of implantations	%	%
	mg	μ u				Fetuses resorbed	Survivors malformed
Retinoic acid	5	1250	9 10 & 11	2	19	95	100
	10	2500		12	112	80	100
Retinyl acetate‡	60	60 000		13	114	54	80
Retinoic acid	5	1250	10 11 & 12	4	37	49	0
	10	2500		4	37	8	100
	20	5000		1	10	70	100
Retinyl acetate‡	60	60 000		11	112	22	83

Only one fetus survived which had severe malformations
 ‡ Data from Hoehhar & Johnson (1965)

Retinoic acid was found quite a potent teratogen also in the rat. Table 2 summarises this data. The treatment period of 9–11 gestational days was very toxic for the embryos. One live fetus out of 19 implants obtained after 5 mg daily (1250 μ u) treatment was severely malformed. It had anencephaly and cleft palate. With a daily dose of 10 mg (2500 μ u) 12 fetuses survived out of 112 implants. 10 of these had sacral spina bifida, 1 had exencephaly and spina bifida and 1 had sirenomelia. Many had a stumpy tail and some had cleft palate as well. Comparing these results with those obtained with retinyl acetate administered over the similar treatment period a dose of 60 000 μ u produced a resorption rate of 54 per cent and 80 per cent of the survivors were malformed with such deformities as exencephaly (15 per cent), cleft palate (80 per cent) and eye malformations (53 per cent).

The treatment period of 10–12 days was much less lethal but equally teratogenic except with the lower dose. With 5 mg daily dose only 1 out of 19 living fetuses obtained was malformed. It was much smaller than its other normal litter mates. It weighed 1.7 gms in contrast to a normal average of 4.0 gms. It also had marked exophthalmos. After a daily treatment with 10 mg retinoic acid only 3 out of 37 implants were resorbed. All the remaining 34 had such malformation as microstomia, open eyes, spina bifida, short tail, many of these with cleft palate. One female given 20 mg retinoic acid daily had 10 implants out of which 7 resorbed. The three living had exophthalmos, 2 of them with spina bifida & micrognathia, 1 also with gastroschisis and 1 with agnathia. Correspondingly a treatment with 60 000 μ u of retinyl acetate during this

gestational period also showed lower resorption rate of 22 per cent. Fifty three per cent of the survivors were abnormal having cleft palate and eye malformations.

DISCUSSION

These results show that retinoic acid is teratogenic at amounts much smaller than what are essential for retinyl acetate to produce malformations. Since biological activities of the two compounds in restoring normal growth in vitamin A deficient animals are reported to be approximately equal (Murray 1962 de Van et al 1961) in order to account for the quantitative differences in their teratogenic activities one must seek some other differences in their biochemical and physiological properties. It has been found (Thompson & Pitt 1961 Thompson 1965) that retinoic acid is much more potent than retinol in inducing signs of hypervitaminosis A in rats. Also its toxicity is much in excess of that of retinol as revealed in organ culture experiments on *Xenopus* larvae (Weissmann Bell & Thomas 1963) and chick limb rudiments (Fill Dingle & Webb 1962) and in incubating chicken eggs (Thompson et al 1965). It is quite probable that the higher potency of retinoic acid as encountered in these experiments also accounts for its increased teratogenic activity.

Some of the previous workers investigating retinoic acid for its ability to ameliorate lesions of vitamin A deficient animals have also observed it to produce effects on their reproductive functions which can be considered to be of a teratological nature. Thompson et al (1964) administered retinoic acid to previously vitamin A deficient pregnant rats in amounts of 1 mg per rat per day throughout pregnancy. No living fetus was obtained; they were found to be in various stages of resorption. This embryotoxic effect was however ascribed to the conditions of vitamin A deficiency still prevailing in the mother which retinoic acid had failed to prevent. Adequate support to this fact was given by control experiments where deficient animals when given plentiful supply of retinol along with retinoic acid cast normal litters. Nevertheless the fact remains that on the basis of observations reported here the amounts of retinoic acid administered to animals by Thompson et al (1964) were sufficient to have caused embryonic death and resorption directly. Parallel findings were also reported in the chick (Thompson et al 1965) where (eggs from vitamin A deficient hens maintained with retinoic acid showed abnormal development of the embryo.

The types of malformations observed with the two agents for the most part were similar both in rat and the mouse. In the rat however spina bifida occurred only after retinoic acid and not after retinyl acetate. This may not be indicative of any significant difference in teratogenic activity since spina bifida has been observed earlier in rats after retinyl acetate (Chouhan 1953 Giroud & Martinet 1956). However the

incidence reported was quite low. Increased incidence of spina bifida may possibly be due to the ability of retinoic acid to produce conditions of severe hypervitaminosis A not possible to achieve with the amounts of retinyl acetate employed in these experiments (Kochhar & Johnson 1965).

For general insights into the mechanism of action of teratogenic agents the use of hypervitaminosis A holds great interest because some of the previously unknown metabolic involvements of vitamin A are gradually coming to light. From a review by Wolf (1962) it is apparent that vitamin A has a profound effect on such tissues as epithelium and cartilage which are characterized by the presence in them of considerable amounts of mucopolysaccharides. Synthesis of mucopolysaccharides by mucus secreting cells of rat colon has in fact been shown to be vitamin A dependant (Wolf & Tarandani 1960). The well known effect of vitamin A on the cartilage in dissolution of mucopolysaccharides from its intercellular matrix is mediated by what has now come to be known as a generalized effect that the vitamin has on the membranes of a number of cells and intracellular organelles (Dingle & Lucy 1963).

Some of these biochemical effects of vitamin A can be experimentally investigated for their possible role in teratogenesis. One study directed to this end (Kochhar & Johnson 1965) has shown alterations in the metabolism of sulfated mucopolysaccharides of mesenchymal tissue and cartilage of malformed embryos following maternal treatment with retinyl acetate.

One shortcoming encountered previously in the use of retinyl esters as teratogenic agents is that once administered they remain in the mother's body too long consequent prolonged exposure of the developing embryo to them interferes in attempts to critically analyze the embryonic stage susceptible to their teratogenic influence. Such an approach may become feasible now since retinoic acid even after administration of high doses rapidly disappears from tissues of animals (Arens & van Dorp 1946; Sharman 1949). Because of this advantage and others mentioned above retinoic acid may prove to be an agent of choice for further studies on hypervitaminosis A induced teratogenesis.

SUMMARY

All trans retinoic acid when administered to pregnant rats and mice produces congenital malformations in their fetuses. This teratogenic response is elicited with a much smaller dose and greater severity than previously reported for other vitamin A compounds. On the basis of biological activity retinoic acid is 40 times as active as retinyl acetate in producing congenital malformations in the mice. Under the conditions employed in the present experiments only retinoic acid produces spina bifida in the rat. It is suggested that the greater teratogenic activity of retinoic acid may be related with its higher toxicity noticed

in earlier studies. It is hoped that comparative study of teratogenic activities of two or more closely related compounds such as retinoic acid and retinyl acetate may eventually lead to understanding of the mechanism of action.

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COMPLEMENT FIXING LIVER ANTIBODIES

*3 Their Serological Relationship to Thyroid Auto antibodies
and Rheumatoid Factors in Patients with Chronic Hepatitis
and in Normal Blood Donors*

By

G NORLP

Received 22 xii 66

The demonstration of serum substances reacting *in vitro* with extracts from various tissues in patients with cirrhosis of the liver has led to the assumption that immunological processes might play a role in the pathogenesis of chronic hepatitis

Non species and non organ specific antibodies against cytoplasmic antigens in liver cells and bile ductular epithellum have been observed by complement fixation using suspensions of various human and animal tissues as antigen (Bjorneboe & Krag 1947 Gajdusek 1958 Deicher et al 1960 Golken 1962 Pasmich et al 1962 Walker et al 1962 Vorup 1967) and by the immunofluorescent antibody technique using cryostat sections of human and animal liver kidney thyroid gland and smooth muscle as antigen (Hunter et al 1960 Paronetto et al 1961 1964 Bumbalo et al 1962 Johnson et al 1964 Whittingham et al 1966 Doniach et al 1966) Cases of active chronic hepatitis associated with a positive L.E. cell test (lupoid hepatitis) have been reported by several investigators (Joske & King 1958 Mackay et al 1956 Andersen & Skjeggstad 1962 Iwaj & Wood 1962) Antibodies to different antigenic components of cell nuclei have been observed with varying frequency in sera from patients with chronic hepatitis using the complement fixation technique (Asherson 1959 Deicher et al 1960) the immunofluorescent technique (Calabresi & Greenberg 1960 Weir et al 1961 Holborow et al 1963 Douchier et al 1964 Faber & Filing 1965 Doniach et al 1966) and the anti gammaglobulin consumption technique (Russel et al 1957 Linke 1965)

In all the studies referred to above positive reactions occurred most frequently in patients with posthepatitic (postural postnecrotic juvenile) cryptogenic (active chronic hepatitis lupoid hepatitis plasma cell hepatitis) and primary biliary cirrhosis whilst in patients with alcoholic or secondary biliary cirrhosis the antidi findings did not differ from those observed in materials of healthy individuals (Mackay & Iarkin 1958 Golken 1962 Vorup 1967)

Antibody to thyroglobulin which is generally assumed to be organ specific and which occurs in 85-90 per cent of sera from patients with Hashimoto's thyroiditis (Routt & Danisch 1958 Hjort 1963) has been observed with varying frequency in patients with chronic hepatitis and in healthy individuals (Table 1) In all the studies referred to in this table no relationship between the incidence of positive TBC tests and antigenological type of chronic hepatitis was found.

Rheumatoid factors a complex of macroglobulins occurring in sera from 5-85 per cent of the patient with rheumatoid arthritis and from 0-8 per cent of normal individuals (Ziff 1957 Wiblad 1960b Serafini et al 1964) have been found with

varying frequency in patients with hepatic cirrhosis in some extent depending on the technique employed. In Table 2 the results obtained with the latex fixation test in chronic hepatitis are summarized. It appears that a positive latex fixation test was frequently found in patients with chronic hepatitis whilst a negative test was observed in most of the cases with biliary cirrhosis.

Using the sensitized sheep cell test (Waller Rose reaction) rheumatoid factor has been reported in 5-20 per cent of sera from patients with hepatic cirrhosis (Harter & Dickgiesser 1953 Winblad 1960b Waldenström *et al* 1964). Studies in which the sensitized sheep cell test has been carried out in parallel with one or more of the so called F II tests (the latex fixation test of Singer & Plot (1956) the bentonite flocculation test of Bo wenich *et al* (1958) the acryl fixation test of Winblad (1960a)) on the same group of sera have shown that the sensitized sheep cell test was positive less frequently than any of the F II tests (Winblad 1960b Holley *et al* 1961 Bouchier *et al* 1964).

TABLE 1
Results of Tanned Red Cell (TRC) Test in Chronic Hepatitis and Healthy Individuals

Authors		Chronic Hepatitis		Healthy Individuals	
		Total Tested	No of +ve	Total Tested	No of +ve
Hackett <i>et al</i>	1960a	24	5	109	9
Shanase & Nilsson	1961	33	1	250	10
Mackay & Wood	1962	59	10	168	7
Bouchier <i>et al</i>	1964	101	10		
Doniach <i>et al</i>	1966	158	41	158§	29

Results of immunofluorescent test for colloid antibody are included
§ Matched for sex and age with the patients

TABLE 2
Results of Latex fixation Tests Carried out on Sera from Patients with Hepatic Cirrhosis of Various Types

Authors		Chronic Hepatitis		Biliary Cirrhosis			
		Total Tested	No of +ve	Secondary		Primary	
				Total Tested	No of +ve	Total Tested	No of +ve
Dresner & Trombly	1959	69	17	7	2		
Howell <i>et al</i>	1960	25	9	8			
Aluater & Jacox	1963	71	41	13	10		
Caplan	1963	99	23	26	1		
Butler & Paton	1964	91	15	17	1		
Walker <i>et al</i>	1965			21	4	31	9

Chiefly of the alcoholic type (Laennec's cirrhosis)

Polish & Muschel (196) examined sera from 29 patients with cirrhosis of the liver (10 with postnecrotic 6 with chronic hepatitis and 13 with alcoholic cirrhosis or fatty liver) for rheumatoid factor using the bentonite flocculation test and for complement fixing antibodies to suspensions of rabbit liver and calf thymus as well as to nucleoprotein derived from calf thymus. Of the 16 cases with postnecrotic cirrhosis and chronic hepatitis rheumatoid factor was found in 7 and complement fixing antibodies to liver and/or thymus tissue in 5. However in no case were rheumatoid factor and complement fixing antibody found to be present in the same

serum. None of the cases with alcoholic cirrhosis had rheumatoid factor although complement fixing tissue antibody was observed in the sera from 4 of them.

Whilst several studies on the frequency of circulating tissue antibodies and rheumatoid factors in materials of patients with chronic hepatitis have been reported only one of the investigations referred to above gives any information about the frequency with which the different antibodies occur in serum from the same patient.

Since it has been shown that circulating complement fixing tissue antibodies are just as characteristic of disseminated lupus erythematosus as are rheumatoid factors of rheumatoid arthritis and antibody to thyroglobulin of Hashimoto's thyroiditis it has been considered of interest to determine the frequency of the occurrence of the above mentioned antibodies in a material of patients with chronic hepatitis and to correlate the incidence of these antibodies in the individual patient.

MATERIAL AND METHODS

Sera from 135 patients with different types of chronic hepatitis were studied. The distribution of the patients with regard to sex and age and to the four aetiological types (posthepatic, cryptogenic, alcoholic and biliary) of cirrhosis has been given in a previous paper (Vorup 1967). In addition a variable number of sera from 194 normal blood donors selected as previously described (Vorup 1965) were examined.

Rheumatoid factors in the sera were demonstrated by 1) the latex fixation test, 2) the sensitized sheep cell test (Wåler Rose reaction) and 3) the streptococcal agglutination test.

1) The latex fixation test (Hyland RA test) was performed according to the manufacturer's instruction. The titre of a serum was read as the reciprocal of the highest dilution causing macroscopically visible agglutination of the latex particles. Titres ≥ 90 were considered positive.

2) The sensitized sheep cell (SSC) test was performed by a modification of the technique described by Heller *et al.* (1949, 1950).

Preparation of anti sheep erythrocyte rabbit serum (amboceptor). Sheep cells for injection were washed three times in saline and a 50 per cent suspension was prepared. The rabbits were immunized according to the following inoculation schedule: two series each of 5 injections of 0.5 ml were given on alternate days with an interval of 7 days. 5 days after the last injection the agglutination titre for sheep erythrocytes was determined in ear blood. If the agglutination titre was sustainable (1:500 or more) the rabbit was bled. After inactivation (56°C for 30 min) the rabbit serum was distributed on small tubes in amounts of two ml and stored at -20°C. No significant decrease in haemagglutinating ability was observed after 6-8 years storage at this temperature. The amboceptor could be thawed and frozen repeatedly (15-20 times) without appreciable titer fall.

Titration of anti sheep erythrocyte rabbit serum. Dilutions of serum ranging from 1:100 to 1:2400 were made up in volumes of 0.5 ml in Wassermann tubes and 0.5 ml of a one per cent suspension of normal sheep cells was added. The tubes were incubated in a water bath at 37°C for 60 mins and subsequently at room temperature (20-22°C) for 10 mins whereafter reading was performed. The reciprocal of the highest initial dilution of amboceptor showing macroscopically visible agglutination of the sheep cells was designated the minimum agglutinating dose (MAD).

Sensitivity of sheep erythrocytes. Sheep cells from defibrinated blood were washed three times in saline whereafter a two per cent suspension was prepared. Equal volumes of this suspension and anti sheep cell rabbit serum diluted to 4 MAD per ml were mixed and incubated in water bath at 37°C for 20 mins. The mixture was stored at 4°C until required. A new portion of sensitized cells was prepared for daily use.

Performance of the Wåler reaction. At 56°C for 30 mins one ml serum was

absorbed with 0.25 ml packed sheep cells twice at room temperature for 40 mins. The serum was diluted two fold in saline in volumes of 0.2 ml starting with 1.5 whereafter 0.7 ml of a one per cent suspension of sensitized cells was added. To a control series comprising the three lowest dilutions of serum 0.2 ml of a one per cent suspension of normal sheep cells was added. After shaking the tubes were incubated in a water bath at 37 °C for 60 mins and at room temperature for 60 mins where after reading was performed. The agglutination pattern was graded with the naked eye as follows: 2 compact agglutinate or 2-6 large clumps; 1 medium sized or small clumps; 0 no agglutination. The titre of a serum was read as the reciprocal of the highest dilution giving a 1 reaction. Titres ≥ 40 were considered positive.

3) *Streptococcal agglutination test (SAT)* was performed according to the routine technique used in the Streptococcal Department of this Institute (Kathak 1949). 0.5 ml of a living culture of haemolytic streptococci SF 130 (Group A type 1) was added to 0.5 ml of inactivated serum diluted two fold starting with 1:20. After incubation in a water bath at 52 °C for two hours the agglutination patterns were read with the naked eye and graded by numbers from 4-0, 4 representing a large coherent disc, 3, 2 and 1 coarse medium sized and small clumps respectively and 0 no agglutination. The titre of a serum was read as the reciprocal of the highest dilution giving a 1 reaction. Titres ≥ 20 (reaction 2) were considered positive.

Tissue antibodies in the sera were demonstrated by 1) the tanned red cell test and 2) the complement fixation test.

1) *Tanned red cell (TRC) test* was mainly performed by the modification of Boyden's haemagglutination technique (Boyden 1951) described by Fulthorpe et al (1961).

Thyroglobulin was prepared from normal thyroid tissue (necropsy) by the method of Derrien et al (1948).

Formalin treatment of sheep erythrocytes was performed by a slight modification of the technique described by Weinbach (1956). Sheep blood in modified Alsever's solution (Bukant et al 1946) was washed three times in saline and then suspended in phosphate buffer pH 7.2 to give a 1 per cent suspension. Equal parts of this suspension and formalin (3 vol-per cent formaldehyde) were mixed and incubated in a water bath at 37 °C for 18 hours. After washing three times in phosphate buffer a three per cent suspension of formalin treated cells was prepared.

Tannin treatment of the cells was carried out by mixing equal parts of a three per cent suspension of formalin treated cells and a tannic acid solution 1:20,000. After incubating in a water bath at 37 °C for 15 mins the cells were washed three times in phosphate buffer and then suspended to produce a three per cent suspension.

Coating with thyroglobulin To a 0.2 per cent (w/v) solution of human thyroglobulin was added a three per cent suspension of formalin and tannin treated cells in equal parts. The mixture was incubated in a water bath at 37 °C for 30 mins and after washing twice in phosphate buffer and once in borate succinate buffer pH 7.5 the thyroglobulin coated cells were suspended in a borate succinate buffer which contained normal rabbit serum in a concentration of one per cent to produce a one per cent suspension of cells.

Performance of test 0.1 ml serum to be tested was absorbed with 0.4 ml of a one per cent suspension of formalin and tannin treated cells at room temperature for 15 mins and after centrifuging titrated in 10 fold dilutions starting with 1:10 in a haemagglutination tray. 0.1 ml dilution of serum was mixed with 0.1 ml of a one per cent suspension of thyroglobulin coated cells. As a control 0.1 ml serum dilution 1:10 was mixed with 0.1 ml of a one per cent suspension of formalin and tannin treated cells. The haemagglutination tray was gently shaken and left at room temperature for 4 hours after which reading was performed. The agglutination patterns were evaluated with the naked eye and graded as follows: 3+ thin carpet of cells covering the bottom and walls of the cup; 2+ smooth mat of cells covering a smaller area of the walls; 1+ thicker mat of cells with a narrow ring around the edge and ~ small disc of cells with a heavy ring around the edge or tight button in the bottom of the cup. The titre of a serum was read as the reciprocal of the highest dilution giving an 1+ reaction. Titres ≥ 5 were considered positive.

2) *Complement fixation test (Cafusek's modification)* (Cafusek 1958) of Donnelly's semi micro method (Donnelly 1951) was used with the modifications described in previous papers (Norup 1965, 1967). The tissue antigens used were sus

sions of normal human biopsy liver normal liver from rhesus monkeys and
 rotoxic thyroid glands obtained at operation. Two MHD of complement were used.
 titre of a serum was read as the reciprocal of the highest serum dilution giving
 more than 50 per cent haemolysis. Titres ≥ 8 were considered positive.

TABLE 3

*Incidence of Rheumatoid Factors and Antibody to Thyroglobulin Distributed
 According to Sex in the Sera from Patients with Chronic Hepatitis
 and Normal Blood Donors*

	Sex	Patients with Chronic Hepatitis			Normal Blood Donors		
		No. of Sera			No. of Sera		
		Tested	Positive	Percent age	Tested	Positive	Percent age
Latex Fixation Test	♂	34	14		123	11	
Positive Titre ≥ 20)	♀	73	29	39.3	66	11	16.8
sensitized Sheep Cell Test (Alar Rose)	♂	27	6		75	1	
Positive Titre ≥ 40)	♀	67	8	11.9	53	2	2.3
Streptococcal Agglutination Test	♂	40	5		124	3	
Positive Titre ≥ 90)	♀	86	5	7.9	64	2	2.6
Antibody to Thyroglobulin	♂	39	1		94	11	
Positive Titre ≥ 5)	♀	88	10	8.7	53	4	6.1

RESULTS

Table 3 shows the results of the serological tests for rheumatoid factor and antibody to thyroglobulin distributed according to sex from the patients with chronic hepatitis and healthy individuals. As stated in this table rheumatoid factors were demonstrated from three to seven times more frequently in the patients than in the controls: the latex fixation test and the SSC test reacting positively in 39.3 per cent (42 out of 107) and 14.9 per cent (14 out of 94) of the pathological sera and in 16.8 per cent (11 out of 66) and 2.3 per cent (three out of 128) respectively of the normal sera. The difference between the positive results in patients and in donors is statistically significant both in the case of the latex fixation test ($\chi^2 = 11.95$, $F=1$, $P < 0.1$ per cent) and the SSC test ($\chi^2 = 12.07$, $F=1$, $P < 0.1$ per cent). A positive SAT occurred in 9 per cent (10 out of 126) of the cases with chronic hepatitis and in 2.6 per cent (5 out of 192) of the donors ($P < 5$ per cent). Neither in the patients nor in the donors were rheumatoid factors correlated with sex (the higher frequency of positive SSC tests and SAT in males with chronic hepatitis was not statistically significant).

I am indebted to cand. polit. S. Olsson and to the Statistical Department, Statens Seruminstitut for making the statistical analyses.

TABLE 4
Results of Complement Fixation with Normal Human Dropsy Liver and Normal Liver of Rhesus Monkeys as Antigen Correlated with Rheumatoid Factors and Thyroglobulin Antibody in Sera from Patients with Chronic Hepatitis and Normal Blood Band Donors

Liver Antibody Titers	Positive Reactions									
	Later Fixation Test				Sensitized Sheep Cell Test				Streptococcal Agglutination Test	
	Patients No	%	Normals No	%	Patients No	%	Normals No	%	Patients No	%
≤ 4	26/74	35	10/184	5	10/63	16	2/193	1	10/87	11
8-32	8/19	42	1/5	-	1/17	6	1/5	-	0/23	0
64-256	3/6	57			1/6	91			0/7	-
≥ 512	1/8				2/8				0/9	-
	47/107	59	11/189	6	14/94	15	3/123	2	10/196	8
									5/192	3
									11/127	9
									9/147	6

Positive ≥ 8

TABLE 5
Influence of Rheumatoid Factors and Antibody to Thyroglobulin Distributed According to the Aetiological Type of Cirrhosis

Type of Hepatitis	Total Tested	Latex Fixation Test			Sensitized Sheep Cell Test			Streptococcal Agglutina- tion Test			Fanned Red Cell Test		
		No. of			No. of			No. of			No. of		
		Positive Titres			Positive Titres			Positive Titres			Positive Titres		
		20	40	80	≥160	/		40	80	≥160	≥160	5-25	≥50
Posthepatic	36	7	3	4	20	5	2	19	1	4	14	4	17
Cryptogenic	37	8	5	2	41	4	1	14	1	2	5	2	8
Alcoholic	15	4	1	1	40	2	13	13	1	1	13	1	7

Of the total of 6 cases with primary (one case) and secondary biliary cirrhosis two had a positive latex fixation test (both were cases of secondary biliary cirrhosis) none of the 6 cases reacted positively in the SSC test SAT nor the TNC test

Thyroglobulin antibody occurred with almost equal frequency in the sera from patients (8.7 per cent) as from donors (6.1 per cent). In the patient group a non significant higher frequency of positive TRC tests was found in the females whilst in the donors a positive TRC test occurred almost equally frequently in either sex.

Table 4 shows the incidence of rheumatoid factor and antibody to thyroglobulin correlated with the incidence of complement fixing liver antibody in the sera from patients with chronic hepatitis and normal donors. A positive latex fixation test occurred in 49 per cent (16 out of 33) of the sera reacting positively and in 35 per cent (26 out of 74) of those reacting negatively to liver antigen. Rheumatoid factor was demonstrable by the latex fixation test in 8 out of 19 sera (42 per cent) having liver antibody titres from 8-32 and in 8 out of 14 sera (57 per cent) with titres ≥ 64 . Rheumatoid factor as indicated by a positive SSC test was found in 4 of 31 sera (13 per cent) with positive liver antibody titres and in 10 of 63 (16 per cent) sera with negative titres. None of 39 sera which fixed complement in the presence of liver antigen and 10 of 87 (11 per cent) sera which failed to do so had a positive SAT ($P=2.5$) a result which agrees well with the findings in the control group (none of five sera with positive liver antibody titres had a positive SAT). No relationship was found between the incidence of thyroglobulin antibody and complement fixing liver antibody.

The incidence of rheumatoid factors and thyroglobulin antibody distributed according to type of chronic hepatitis is shown in Table 5. In all types of chronic hepatitis rheumatoid factor was demonstrated more often by the latex fixation test than by the SSC test and SAT. No difference between the incidence of rheumatoid factors in the post-hepatic and cryptogenic group on the one hand and in the alcoholic group on the other was observed. There was no correlation between high titres of rheumatoid factor and type of chronic hepatitis. Thyroglobulin antibody occurred in 17 per cent (6 of 36) of the cases with posthepatic cirrhosis and in 7-8 per cent of those with cryptogenic and alcoholic cirrhosis. In most of the cases the titres were low (<25), only one case of cryptogenic cirrhosis and myxoedema had a titre of 2500 and had also organ specific complement fixing thyroid antibody (titre = 32).

Table 6 shows the incidence of tissue antibodies and rheumatoid factors distributed by age in 94 patients with chronic hepatitis and 129 normal donors. It can be seen that with the exception of the SSC test and SAT the incidence of positive tests had one maximum in patients aged 41-50 years and another one in those aged 61-70 years.

In comparing the results of the serological reactions carried out on the patients and the donors the different age distribution of the individuals in the two materials must be taken into consideration since tissue antibodies and rheumatoid factors have been found more often in elderly than in younger individuals. The difference between the frequencies of positive tests in patients and in donors is however too

TABLE 6
Positive Serological Reactions Distributed on Age of 94 Patients with Chronic Hepatitis and 122 Normal Blood Bank Donors

Age	No. of Subject Patients	Complement fixing			Positive Cases			Sensitized Sheep Cell Test			Streptococcal Agglutination Test			At Least One of the Antibodies Present		
		Thyroid Antibody			Thyroid globulin Antibody			Fixation Test			Agglutination Test			Agglutination Test		
		patients	donors	ratio	patients	donors	ratio	patients	donors	ratio	patients	donors	ratio	patients	donors	ratio
40-49	4	7	(5)	0.7	10	0	0	50	53	(25)	13	(0)	4.0	(50)	10.0	
50-59	13	7	5.0	3.8	15.4	0	0	41.7	11.5	16.6	0	0	0	75.0	19.2	
60-69	20	17	13.6	5.9	4.8	5.3	37.3	11.8	18.2	5.9	9.1	1.0	50.0	9.4		
70-79	34	4	38.9	(95)	0.6	(0)	14.7	(15)	41.9	(25)	11.8	(93)	14.7	(0)	76.5	(25)
80-89	71	2	27.3		13.6		9.1	45.5		13.6		1.1		13.6		
Total	94	122	33.0	4.1	17.0	4.1	10.1	29.4	8.9	14.9	9.5	9.6	3.1	66.0	21.7	

27 males and 67 females 72 males and 50 females

great to be accounted for by this shift in the age distribution for example the incidence of complement fixing liver and thyroid antibody was significantly higher in the patients than in the donors ($P < 0.1$ per cent). In addition a non negligible percentage of positive reactors in the donor group was under 40 years a fact which may reduce the possible error resulted from the different age distribution of the two materials 66.7 per cent (62 of 94) of the sera from patients with chronic hepatitis

TABLE 7

Relationship between the Occurrence of Complement fixing Liver and Thyroid Antibody and Rheumatoid Factors as Determined by Various Techniques in the Sera from 32 Patients with Chronic Hepatitis

Serial No	Age (yr)	Sex	Complement Fixation		Rheumatoid Factors		
			Normal Human Biopsy Liver	Thyrotoxic Thyroid Glands	Latex Fixation Test	Sensitized Sheep Cell Test	Streptococcal Agglutination Test
1	64	♀	4096	2048	80	40	
2	69	♀	4096	2048			
3	65	♀	2048	2048	20		
4	60	♀	512	128			
5	57	♀	206	128			
6	50	♀	128	8			
7	49	♀	64	32			
8	70	♂	64	16			
9	85	♀	64	16	20		
10	72	♀	32	16			
11	49	♀	32	16	20		
12	58	♀	1024		20		
13	37	♀	1024		160	160	
14	62	♂	512		20		
15	55	♀					
16	44	♀	128		320	40	
17	75	♀	32		20		
18	76	♀	32				
19	64	♂					
20	67	♂					
21	67	♂	8		40	40	
22	80	♀		8	20	40	
23	46	♂			40	40	
24	54	♀			20	40	
25	69	♀			20		20
26	69	♀					
27	67	♂			1280	640	160
28	56	♂			640	320	160
29	76	♂			160	20	160
30	59	♂			160	40	80
31	66	♀			80	80	160
32	76	♀			20	40	160

All sera were tested for thyroglobulin antibody using the tanned red cell test. Only one (case no. 7) serum reacted positively (titre 1:25).

and 21.3 per cent (26 of 122) of the controls had at least one of the antibodies shown in Table 6. The difference in positivity between patients and donors was significant ($P < 0.1$ per cent).

Table 7 shows the incidence of complement fixing tissue antibody correlated with rheumatoid factor in the sera from 32 patients with chronic hepatitis. It will appear from the table that sera which fixed complement in the presence of liver and thyroid tissue rarely had rheumatoid factor whilst on the other hand sera in which rheumatoid factor was demonstrable by at least two of the three tests rarely had complement fixing tissue antibody. Of the 20 cases (Nos 1-20) which had complement fixing liver antibody at titres ≥ 32 three (Nos 1, 13, 16) reacted positively both in the latex fixation test and the SSC test. All three cases were females, two of them had posthepatic cirrhosis (Nos 13, 16), one had cryptogenic cirrhosis (No 1). Low titres ($=20$) of rheumatoid factor was demonstrated by the latex fixation test in 7 patients (one male and six females). Of these 4 (Nos 3, 12, 14, 15) had complement fixing liver antibody at titres ≥ 512 and three (Nos 9, 11, 17) had titres of 32-64. Of the 6 sera (Nos 21-26) in which rheumatoid factor was demonstrable by two of the three tests, one (a male with cryptogenic cirrhosis) had complement fixing liver antibody and one (a female with posthepatic cirrhosis) had complement fixing thyroid antibody, in both cases the titre was 8. In the sera from 6 patients (Nos 27-32) rheumatoid factor was demonstrable by all three tests. In none of the six sera complement fixing antibody to liver and thyroid tissue were present. It is noteworthy that some of the most hightitred sera of the whole material could be found in this patient group and that 4 of the 6 patients were males. Three (Nos 27, 29, 31) of the six cases had posthepatic cirrhosis, one (No 28) had cryptogenic cirrhosis and two (Nos 30, 32) had alcoholic cirrhosis.

DISCUSSION

The presence of circulating tissue antibody associated with a marked hypergammaglobulinemia which can be seen in certain forms of chronic hepatitis has been assumed by some investigators (Havens 1959, 1963; Polish & Vusichel 1962) to be evidence of an enhanced activity of the reticulo-endothelial system. In accordance with this assumption patients with chronic hepatitis have been found to respond more vigorously to a booster injection of purified diphtheria toxoid than patients without chronic hepatitis (Havens *et al.* 1951).

The frequent occurrence of rheumatoid factors in chronic hepatitis which has been reported by earlier investigators may lend further support to the above mentioned concept (Dresner & Trombly 1959; Howell *et al.* 1960; Atwater & Jacob 1963; Kaplan 1963; Butler & Paton 1964; Bouchier *et al.* 1961).

The results of the present investigation have confirmed that rheuma-

toid factors occur with higher frequency in chronic hepatitis than in healthy individuals and that the latex fixation test reacts positively more often than the SSC test and the SAT. In the present study no difference between the incidence of positive latex fixation tests and SSC tests in the cases of alcoholic cirrhosis on the one hand and in those of posthepatic and cryptogenic cirrhosis on the other was observed.

In this connection it is of interest that complement fixing liver antibody occurred almost exclusively in the cases of posthepatic and cryptogenic cirrhosis (Norup 1967) a result which may suggest that different immunological mechanisms take part in the formation of rheumatoid factors and complement fixing liver antibody in chronic hepatitis.

Only few investigations using the streptococcal agglutination test in chronic hepatitis have been undertaken. In a larger material of hospitalized patients with various medical diseases excluding rheumatoid arthritis Kalbak (1948) found a positive SAT in 7 per cent (36 of 500) compared with 1.5 per cent of the sera from 64 healthy blood donors. Similarly in the present investigation 7.9 per cent (10 of 126) of the sera from patients with hepatic cirrhosis and 2.6 per cent (5 of 192) of the donors had a positive SAT.

The occurrence of thyroglobulin antibody in patients with chronic hepatitis has been assessed variously in different laboratories obviously owing to differences in the technique and antigens used. Thus Mackay & Wood (1962) using a thyroid antigen for coating the tanned cells have observed positive TRC tests in 19 per cent (10 of 52) of cases of chronic hepatitis whilst Skanse & Nilsson (1961) who used purified thyroglobulin as antigen found a positive TRC test in 3 per cent (1 of 33) of sera from patients with chronic hepatitis. In the present investigation thyroglobulin antibody was found with almost equal frequency in patients (8.7 per cent) as in donors (6.1 per cent). In this connection it should be mentioned that of 100 sera from patients with rheumatoid arthritis only 4 had antibody to thyroglobulin (Norup unpublished data).

Several investigations have been carried out with regard to the relationship between the incidence of tissue antibodies and sex and age. It seems apparent from most of these investigations that non-organ specific tissue antibody are correlated to sex occurring more frequently in females than in males but not to age (Hackett *et al* 1960b, Kalas *et al* 1963, Serafini *et al* 1964) and that organ specific tissue antibodies are correlated to both sex and age having been found more frequently in elderly women than in elderly men (Coudie *et al* 1959, Hackett *et al* 1960a, Hill 1961, Serafini *et al* 1964). In the present series of patients complement fixing thyroid antibody occurred significantly ($P < 0.05$ per cent) more frequently in females than in males and a non significant increase of positive TRC tests in females aged 61-70 years was observed.

Where the relationship of rheumatoid factor to sex and age is concerned the results are not consistent although studies carried out on large materials of sera from healthy individuals have suggested an increasing incidence of rheumatoid factor with increasing age in either sex (Ball & Lawrence 1961; Serafini *et al* 1964).

The poor correlation between the occurrence of rheumatoid factor and complement fixing tissue antibody having been found in the present series of patients is at present difficult to interpret. In this connection it is of interest that a co-existence of rheumatoid factor and complement fixing tissue antibody in patients with disseminated lupus erythematosus was not encountered more frequently than might be expected by chance: thus of 37 patients 11 (30 per cent) had complement fixing tissue antibody, 9 (24 per cent) had rheumatoid factor as demonstrated by the latex fixation test and only 7 (19 per cent) had both complement fixing tissue antibody and rheumatoid factor (Norup unpublished data).

The findings here presented seem to indicate that patients with chronic hepatitis produce gammaglobulins with rheumatoid factor activity in their reticulo-endothelial system in amounts exceeding those which can occasionally be found in apparently healthy individuals. Although the causes of this enhanced production of rheumatoid factor like globulins and moreover of antibodies to constituents of various tissues are not known their presence in serum seems to suggest that the primary lesion causing chronic hepatitis may be sought in the reticulo-endothelial system of the patient.

SUMMARY

Sera from patients with various types of chronic hepatitis and from healthy individuals were examined for rheumatoid factors using the latex fixation test, the sensitized sheep cell (SSC) test and the streptococcal agglutination test (SAT) and for thyroglobulin antibody using the tanned red cell (TRC) test.

A positive latex fixation test was found in 39.3 per cent (42 of 107) and 5.8 per cent (11 of 189), a positive SSC test in 14.9 per cent (14 of 94) and 2.3 per cent (3 of 128) and a positive SAT in 7.9 per cent (10 of 126) and 2.2 per cent (5 of 192) of the sera from patients with chronic hepatitis and from healthy blood donors respectively.

No correlation was found between the incidence of rheumatoid factors and aetiological type of hepatic cirrhosis.

By correlating the occurrence of rheumatoid factor and complement fixing tissue antibody a positive latex fixation test occurred in 49 per cent (16 of 33) of the sera reacting positively and in 35 per cent (26 of 74) of those reacting negatively to liver antigen, a positive SSC test in 13 per cent (4 of 31) of the liver positive sera and in 16 per cent (10 of 63) of the liver negative sera. Neither in patients nor in donors did

a positive SAT occur together with complement fixing liver or thyroid antibody

The incidence of thyroglobulin antibody in patients with chronic hepatitis did not differ from that found in the donors

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SERUM P_i TYPES IN NORWEGIANS

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The term P_i proteins was given to a system of serum proteins migrating faster than albumin during electrophoresis in acidic starch gels (4, 5, 6). The P_i proteins were found in five different phenotypes fitting a genetic theory of three codominant alleles called PⁱPⁱ, PⁱM and PⁱS.

It has recently been shown (6) that the P_i proteins correspond to α_1 antitrypsin. In ordinary starch gel electrophoresis α_1 antitrypsin migrates with the albumin. When the pH in the gel is lowered to about 5, a series of protein zones—the P_i proteins—carrying the α_1 antitrypsin antigenic determinant appear in front of the albumin.

The present report deals with the P_i phenotypes and P_i gene frequencies in Norwegians and the discovery of two new alleles.

MATERIALS AND METHODS

Human sera. Sera were obtained from 1043 healthy blood donors from the Red Cross Transfusion Centre Oslo and from 780 healthy blood donors and 1007 pregnant women from The Regional Transfusion Centre Stavanger Norway. In addition blood samples were collected from 24 members of four families where new phenotypes were observed. Only one serum sample from each person in the Stavanger material was tested. Sera giving other P_i band patterns than M_iM_i were retested at least once. In the Oslo material these persons were also retested after new bleedings. The 390 blood donors typed by Fagerhol & Brøn I (4) are not included in this material. The sera were stored below -20°C until tested.

The donors at the Red Cross Transfusion Centre Oslo are predominantly citizens of Oslo but some are living in the suburbs. Likewise most of the blood donors in Stavanger live in that city. The transfusion centre in Stavanger receives blood samples for antenatal Rh blood typing and antibody screening from pregnant women living in south western Norway mostly from Stavanger and the county of Rogaland. Since it has been shown (3) that the genes determining the α_1 antitrypsin types are not sex linked the blood donors and the pregnant women from Stavanger and Rogaland are treated together and referred to as the Stavanger population.

Starch gel electrophoresis. A modification of Poulik's (8) horizontal discontinuous system was used with pH 4.95 in the gels. The P_i typing is based upon the migration rates and the appearance especially the staining intensity of the P_i protein zones in front of the albumin.

Conventional statistical methods were used. The gene frequencies were found by direct counting of genes.

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RESULTS

A total of twelve different P_i phenotypes were observed. A schematic drawing of them is presented in Fig. 1. In the drawing the band patterns are stretched out to illustrate the relative positions of the protein zones in the different phenotypes. The relative staining intensities are indicated by hatching: increasing from white through hatched, cross hatched and heavily hatched, to black.

The phenotypes FM, MM, MS, FS and SS have been described earlier (4). By technical improvement it has been possible to separate completely the F zones from the M zones in the FM phenotype as can be seen in Figs. 1 and 3. The present study disclosed five new phenotypes which will be designated FF, IM, IS, SZ and MV respectively.

The phenotype called FI, found in one person, consists of one minor and two major protein zones of about the same staining intensities as the corresponding MM zones. But the FI zones have higher migration rates and the distances between the zones are slightly shorter than between the MM zones. In Fig. 2 is shown that the parents and the two children of the FI propositus all have the FM phenotype. It is therefore concluded that this propositus is homozygous $P_i^F P_i^F$.

In the phenotypes IM and IS protein zones corresponding to the MM and SS zones are found respectively but with about half their staining intensities. The IM and IS band patterns have three zones in common.

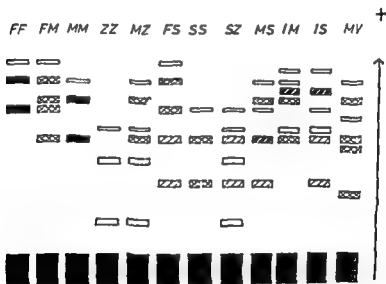


Fig. 1

Schematic drawing of the twelve P_i phenotypes in front of the albumin. Staining intensities are indicated by different shadings of black: increasing from white through hatched, cross hatched, heavily hatched to black.

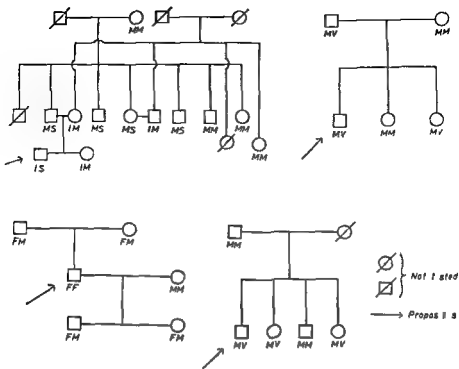


Fig. 2

P_i phenotypes observed in four families where new phenotypes were found

which are specific for these phenotypes: one very weak zone in front, one relatively strong zone (zone no. 2 in the IS phenotype) and one weak zone closely in front of the slowest MM zone. The relative positions of these three zones correspond to the MV band pattern except that their migration rates are intermediate between those of the FF and MM protein zones. In Fig. 2 is shown the P_i phenotypes observed in the family of the IS propositus. Protein zones corresponding to the intermediate band pattern were found in three of his relatives. It is therefore concluded that the band patterns IM and IS are heterozygous phenotypes between a new allele for which the term P_i^I is proposed and the alleles P_i^M and P_i^S respectively. The general pattern of the allele products is one weak zone in front of two major zones of about equal staining intensity. It is evident that P_i^I allele product deviates from this general pattern since the slow major zone is much weaker than the major zone in front.

The phenotype called MV consists of two weak and four major zones with about half the staining intensity of the MM zones. The two major zones in front correspond to the MM zones while the two major zones behind have lower migration rates than the SS zones. The distance between the two slowest MV zones is slightly longer than between the



Fig 3

Photograph of an amido black stained starch gel showing the P_i phenotypes IM and FM

major SS zones. In Fig. 2 is given the P_i phenotypes found in two unrelated families where the phenotype IM was observed. It is concluded that the IM phenotype is a new heterozygous type resulting from the combination of the allele P_i^M and a new allele for which the term P_i^N is proposed.

It has been shown (6) that sera from persons who are homozygous for α_1 antitrypsin deficiency give P_i band patterns with slower migration rate than the V band pattern. In addition the protein zones are very weak corresponding to about 10 per cent of the normal α_1 antitrypsin concentration in serum. The term P_i^Z was therefore chosen for the allele for α_1 antitrypsin deficiency (6). Only when at least 5 times the normal amount of serum is applied in the starch gel for P_i typing, can the ZZ zones be seen. Even then the protein zones are weak. The sera which on repeated testing (using normal amounts of serum) gave no clearly visible protein zones in the P_i region were classified as phenotype ZZ. Those sera which on repeated testing gave MM or SS zones with about half the normal staining intensities as judged by the unaided eye were classified as MZ and SZ respectively.

Fig. 3 is a photograph of an amido black stained starch gel where the phenotypes FM and IM are compared and in Fig. 4 is shown the new phenotype MV compared with MM, MS and SS. On this photograph the weak zone belonging to the V band pattern can not be seen as it disappears between the M zones. The optimal conditions for separation of the partially overlapping phenotypes IM and FM are somewhat dif-



Fig. 4

Photograph of an amido black stained starch gel where the phenotypes SS MS MM and MV are compared

ferent from the optimal conditions for slow heterozygous types as MV. The gels shown on these two photographs were therefore run under somewhat different conditions as can be seen from the positions of the albumins.

The protein zones resulting from Pi^S are much weaker than those resulting from the other alleles except Pi^Z . This observation has been made on all SS MS 1S and FS phenotypes studied. Quantitative data are not yet available but studies in this respect are in progress.

In Table 1 is given the distribution of Pi phenotypes in 1781 persons living in the Stavanger area and 1013 persons living in the Oslo area and in the total of 2830 Norwegians. As can be seen the observed and expected number of individuals of the different phenotypes are quite similar. In Table 2 is given the gene frequencies found in the same populations. It is evident that the gene frequencies found in Stavanger population do not differ significantly from those in the Oslo population. These two populations were therefore added and the gene frequencies calculated for the total material of 2830 Norwegians.

TABLE
Observed and Expected P_i Phenotypes in 1787 Persons Living in the Stavanger Area

		MM	MS	FM	MZ	IM	FF	IV
Stavanger	Observed	1600	80	47	44	5	1	1
	Expected	1595	85.1	46.97	49.97	4.798	0.3354	1.013
Oslo	Observed	940	36	25	37	1	0	1
	Expected	939.6	38.01	24.75	35.04	1.98	0.163	0.99
Total	Observed	2540	116	72	81	6	1	2
	Expected	2534	123.2	71.74	84.09	6.427	0.5006	2.144

TABLE 2
Gene Frequencies Observed in the Stavanger and Oslo Populations and in a total of 2830 Norwegians

	P_i^M	P_i^S	P_i^Z	P_i^F	P_i^I	P_i^N
Stavanger	0.9449	0.0757	0.0145	0.0137	0.0014	0.0003
Oslo	0.9491	0.0192	0.0177	0.0125	0.0010	0.0005
Total	0.9463	0.0230	0.0157	0.0133	0.0012	0.0004

DISCUSSION

It is seen from Table 2 that the P_i system consists of one common allele P_i^M and several more or less rare alleles. According to the genetic theory 21 different phenotypes are expected for six alleles but only twelve have been found yet. The allele P_i^N described by Axelsson & Laurell (1965) and retested by Tagerhol & Laurell (1967) were not found in this study. From the gene frequencies it is seen that the phenotypes P_i^I , P_i^N , P_i^Z , P_i^I , P_i^S , P_i^I and P_i^Z must be very rare in Norwegians and none of these were observed. The phenotype P_i^Z was not found but this is probably a matter of chance since the expected number was only 1.1182.

Axelsson & Laurell (1965) have presented evidence including family studies that P_i^Z and P_i^N are alleles to P_i^M . The distribution of phenotypes as well as the family studies presented in this report support the genetic theory advanced (4) that the P_i system consists of several codominant alleles. Exceptions to this mode of inheritance have not been observed.

Several authors (2, 7, 9) have found that α_1 antitrypsin is unstable to heat and low pH. The observation that the P_i zones carrying the antigenic determinant of α_1 antitrypsin appear in front of albumin as the pH is lowered towards 5 in starch gel electrophoresis suggests that the P_i zones represent split products of α_1 antitrypsin. If this is true

in 1043 Persons Living in the Oslo Area and in the Total of 2530 Norwegians

SZ	SS	FS	IS	ZZ	FZ	χ	n	P
4 1 306	3 1 135	II 1 234	0 0 1261	2 0 3757	0 0 71	3 15	4	0.6 > P > 0.5
0 0 7089	1 0 3845	1 0 5006	1 0 0401	II 0 3268	II 0 4615	0 27	4	0.99 > P > 0.99
4 2 044	4 1 497	1 1 731	1 0 1567	2 0 6976	0 1 182	3 15	5	0.7 > P > 0.6

the protein corresponding to Pi^I must be split into two major polypeptides of unequal molecular weights while splitting of the other allele products gives major polypeptides of about the same size. The chemical basis of the different Pi phenotypes is not known but might well depend upon substitution of one or more amino acids. The difference in staining intensity of the major zones resulting from Pi^I suggests that there may be more than one point of substitution in the protein molecule.

Eriksson (3) has shown that there is a close relationship between the phenotype ZZ (homozygous state of α_1 antitrypsin deficiency) and chronic obstructive bronchopulmonary disease. Whether other Pi phenotypes may predispose for disease remains to be studied.

SUMMARY

Serum Pi types were studied in 2830 Norwegians by starch gel electrophoresis. Twelve different phenotypes were observed fitting a genetic theory of six codominant alleles called Pi^F , Pi^I , Pi^M , Pi^S , Pi^V and Pi^Z . Band patterns corresponding to the new alleles Pi^I and Pi^V are described. The symbols for the allele products were chosen according to the migration rates of the proteins which decrease in the following order: F, I, M, S, V and Z. In this material the following gene frequencies were found: Pi^M 0.9463, Pi^S 0.023, Pi^Z 0.0157, Pi^F 0.0133, Pi^I 0.0012, Pi^V 0.0004. The allele products of Pi^F , Pi^M , Pi^S and Pi^V appear as a three band pattern: one weak zone in front of two major zones. The alleles Pi^Z and Pi^I give exceptional patterns. Pi^Z gives only about 10 per cent of the normal protein concentration while Pi^I gives major zones of unequal staining intensities.

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EXPERIMENTAL STUDIES ON BOVINE TICK BORNE FEVER

1 *Clinical and Haematological Data, some Properties of the Causative Agent and Homologous Immunity*

By

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Received 20 vii 66

Tick borne fever (reviewed by Tuomi (17)) was first described as a disease of sheep; later the condition was also detected in cattle. In the main, earlier studies of both ovine and bovine tick borne fever were made in Great Britain. The occurrence of bovine disease has additionally been reported from Ireland, Norway, the Netherlands and Finland. To date, the only major experimental study of bovine tick borne fever published has been the original paper by Hudson (11).

In Finland, experimental and field studies of bovine tick borne fever have been undertaken since summer 1964. The findings in epidemiological field studies have already been reported (17), as have those of an electron microscopic examination of the structure of the causative agent which led to the suggestion that this agent occupied a taxonomic position in the middle ground between typical rickettsiae and organisms of the psittacosis group (20). The present series of papers contains reports on most of the experimental findings made so far. The first paper describes clinical features in general, the occurrence of tick borne fever, haematological data, carrier state, immunity to homologous strain, preservation of the agent and studies concerned with its cultivability and host range. The subjects of the following papers are: strain differences in virulence, strain differences in immunological properties, the related character of strains as revealed by immunofluorescence technique and the susceptibility of the agent to antibiotics and sulphonamides.

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smears from the yolk sacs and occasionally from other membranes as well from embryo tissues and from blood cells of developing and hatched chickens. From five series of eggs each at a different level of blind passage yolk sac suspensions in sucrose PG (1) were inoculated into susceptible cattle.

Primary calf kidney cells maintained in a medium (Fagle's minimum essential medium +2.5 per cent calf serum +70 per cent tryptose phosphate broth) devoid of antibiotics were once inoculated with blood infected by J33 f4 strain. At 14 days a mixture of cells and medium was inoculated intravenously into a susceptible cow. HeLa and HS-C cells were inoculated once and a blind passage was carried out.

RESULTS

Clinical Features

The following description of clinical features is based upon reactions occurring in animals with no previous experience of tick borne fever. No more than partial reference is made to strain differences as regards the virulence.

Table 1 lists some data in respect of 36 cattle and of their reactions to the tick borne fever agent.

In 35 cattle the incubation time varied from 4 to 11 days with a mean of 6.9 days. As is observable from Table 1 several inocula had been stored in different ways before injection; this procedure often obviously prolonged the incubation time. In cow 2^o on which nymph of *I. ricinus* transmitting the J Puhe strain had been allowed to engorge the incubation period was 12 days. The most conspicuous clinical sign was fever. The recorded maximum temperatures in 36 cattle ranged from 39.7 C to 41.8 C (mean 41.0 C). The form of the fever curve was mostly rather even with a rapid rise to the maximum and a gradual diminution; however, times great fluctuation was evident (Fig. 2). The duration of fever ranged from 1 to 5 days (mean 3.5 days). Secondary temperature rises lasting from one to two days appearing 3 to 8 days after subsidence of the primary reaction were recorded in 9 out of 36 animals.

Concurrently with the fever cattle showed other signs of illness although as a rule these were disproportionately mild in comparison with the high fever. Most animals appeared slightly to moderately depressed with diminished appetite but only during the height of the temperature reaction. As a rule the respiratory rate increased but only exceptionally to more than twice the normal. The pulse rate was only slightly elevated as a general rule. All but two of 20 cattle kept under close observation at the State Veterinary Medical Institute were found to cough during the illness. Cows 21 and 22 which did not exhibit this symptom reacted to the less virulent J Puhe strain (cf. next paper of the series (18)). Several of those kept outside Helsinki were also reported to have coughed (coughing ceased to be delayed when taken against the fever reaction; it started during its latter part or after it had subsided). Coughing was usually mild, dry and infrequent; it could frequently have easily escaped notice if not made the object of specific observation. Only occasionally did coughing last longer than for two three days and then usually became louder as it progressed. Simultaneously with coughing, some animals had discharges from the eyes and nose. Loosening of the consistency of the faeces was noticed in two animals. Two adult cows stood with curved back and frequently lifted their hind feet.

In all the eight cows lactating at the time of reaction there took place a drop in the milk yield ranging from slightly less than half of the previous level. In only one cow was the daily yield measured. A fall from 17 l to 9 l was concurrent with the temperature reaction. The former level was regained in 5 days. In all cows at least nearly full milk production was restored within 2 weeks. Not one of the pregnant cows or heifers aborted in connection with tick borne fever and all of them later had a normal parturition in the due time.

Table 1 indicates that adult cattle tended to be affected more markedly than calves although all the calves even that one week old exhibited a febrile reaction.

Designation of the animal	Age	Seed inoculated			Incubation time days	Temperature reaction		Onset of fever		Maximum percentage of granulocytes showing bodies	
		Strain of agent	Way of storage	Length of storage		Max temp °C	Duration		Primary days		Secondary days
							Primary days	Secondary days			
Calif 3	3 m	J34 64	+4 C	2 d	5	40.5	3	4		22	
Calif 4	3 m		74 C	3 d	5	40.9	4	4		0	
Calif 5	3 m			1 mo	6	40.8	4	5	4	30	
Calif 6	3 m	J34 64	+4 C	3 d	7	40.3	2	3		10	
Calif 7	3 m	J34 64	+4 C	3 d	7	41.0	3	4	1	42	
Calif 8	3 m	J34 64	+4 C	3 d	7	40.4	3	4	1	15	
Calif 9	3 m	J34 64	+4 C	3 mo	8	41.5	3	4	1	38	
Calif 10	3 m	J34 64	+4 C	4 1/2 mo	10	41.0	2	3	1	16	
Calif 11	3 m	J34 64	+4 C	3 d	6	41.6	3	3	1	12	
Calif 12	3 m	J34 64	+4 C	1 mo	7	40.5	3	3	1	11	
Calif 13	3 m	J34 64	+4 C	1 mo	11	40.7	5	4		31	
Calif 14	3 m	J34 64	+4 C	2 1/2 mo	7	41.5	3	4	1	33	
Calif 15	3 m	J34 64	+4 C	3 mo	4	41.1	4	3		48	
Calif 16	3 m	J34 64	+4 C	3 mo	7	41.7	3	4	ne	30	
Calif 17	3 m	J34 64	+4 C	3 mo	7	41.5	3	4		60	
Calif 18	3 m	J34 64	+4 C	3 mo	7	41.8	3	4		58	
Calif 19	3 m	J34 64	+4 C	3 mo	7	40.7	3	4		40	
Calif 20	3 m	J34 64	+4 C	3 mo	7	41.4	2	4		70	
Calif 21	3 m	J34 64	+4 C	3 mo	7	41.4	2	4		48	
Calif 22	3 m	J34 64	+4 C	3 mo	5	41.2	4	2		7	
Calif 23	3 m	J34 64	+4 C	3 mo	12	39.7	2	2		4	
Calif 24	3 m	J34 64	+4 C	3 mo	12	41.2	3	2	2	18	
Calif 25	3 m	J34 64	+4 C	3 mo	10	40.6	7	7		6	
Calif 26	3 m	J34 64	+4 C	3 mo	5	40.9	5	5		16	
Calif 27	3 m	J34 64	+4 C	3 mo	8	40.3	3	3		10	
Calif 28	3 m	J34 64	+4 C	3 mo	7	40.8	4	2		2	
Calif 29	3 m	J34 64	+4 C	3 mo	10	40.6	4	1		50	
Calif 30	3 m	J34 64	+4 C	3 mo	5	41.1	4	5		78	
Calif 31	3 m	J34 64	+4 C	3 mo	5	41.1	4	5	ne	44	
Calif 32	3 m	J34 64	+4 C	3 mo	6	40.5	5	4		53	
Calif 33	3 m	J34 64	+4 C	3 mo	6	41.7	5	4	ne	70	
Calif 34	3 m	J34 64	+4 C	3 mo	7	41.7	4	5	1	72	
Calif 35	3 m	J34 64	+4 C	3 mo	7	41.5	3	6	1	32	
Calif 36	3 m	J34 64	+4 C	3 mo	6	41.4	5	4	2	48	
Calif 37	3 m	J34 64	+4 C	3 mo	7	40.8	4	3			

† = not further examined
§ = blood from the spleen case
ne = not examined

= splenectomized

g = blood from the field case

† = not further examined

ne = not examined

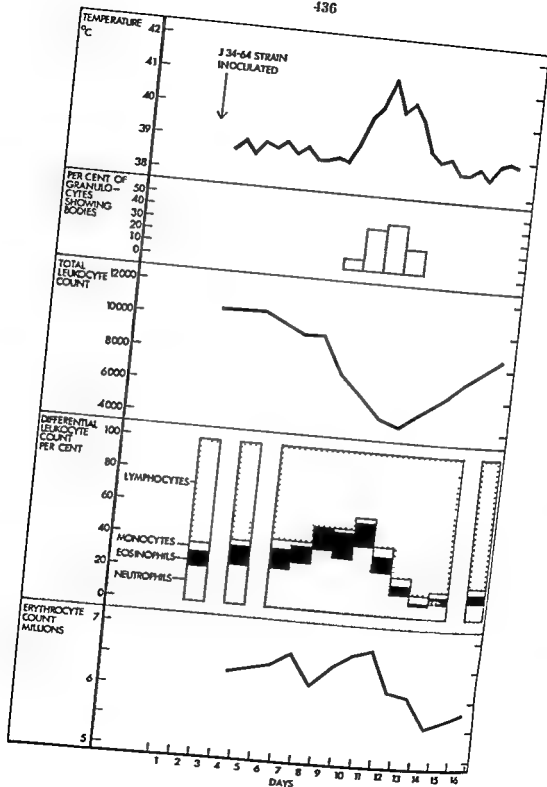


Fig. 3

end of the temperature reaction the number of lymphocytes again began to rise but the diminution in granulocytes continued. At the point when examinations were ended a rise in the number of granulocytes had also commenced. As regards the granulocytes the numbers of both neutrophils and eosinophils seemed to be affected in the same way. No effect on the basophil numbers was observable. Immature neutrophils and even eosinophils were often detected during the diminution phase of granulocyte numbers. At one point in cow 17 the number of band neutrophils reached almost half the number of the mature ones. A slight absolute but not relative rise in the number of monocytes is indicated as having occurred at the end of the clinical reaction and lasting for a few days. A corresponding and some times more marked increase in the number of monocytes was encountered in several other cattle examined. The other findings of these unpublished examinations resemble in principle those presented in Fig. 4. It is further seen from the same Figure that the erythrocyte count fell slightly after the end of the clinical reaction.

Carrier State

The subinoculation of fresh blood obtained during the period in which tick borne fever bodies were detected microscopically regularly resulted in tick borne fever reaction in susceptible animals. With a view to determining the length of the period during which blood usually remained infective after the microscopical disappearance of bodies there were undertaken tests indicated in Fig. 5.

In all the animals except calf 2 which had also reacted to another strain the preceding reaction had been caused by J34-64 strain. In every case but one 3 to 6 ml of fresh blood was inoculated intravenously; however the positive reaction of the blood from heifer 13 obtained 9 days after the disappearance of bodies resulted from the inoculation of 500 ml of blood; this was furthermore the only subinoculation which brought about tick borne fever reaction. All the other tests with blood taken between 3 days and 8½ months after the reaction were negative as were the two splenectomies.

There was no specific intention of studying the carrier state after reactions caused by other agent strains. Nevertheless on one occasion such a state was encountered accidentally. The blood of sheep 2 (splenectomized animal) was found to carry J-Puke strain after the elapse of approximately one month and one week from subsidence of the clinical reaction caused by this strain. This finding will be described in greater detail in one of the later papers (19). Later papers will also show that J-Puke strain is highly virulent in sheep (18, 19).

Immunity to Homologous Strain

An examination was made of the development and duration of homologous immunity for 6 various strains in both cattle and sheep by the application of challenge tests. The results obtained are presented in Fig. 6.

Most tests were concerned with immunity against J34-64, J-Puke and K70-65 strains. On none of the 30 occasions in which the first challenge was effected within 3 months (the majority were within 3 weeks) after subsidence of the preceding reac-

Fig. 5

A composite chart of temperature reactions and occurrence of tick borne fever bodies and haematocrit in three cattle (cows 17, 18 and 20) inoculated simultaneously with J34-64 strain. The curves are means from the three animals.

Preservation of Infectivity in Stored Blood

In a number of tests it proved that the infectivity of blood obtained at the height of a primary tick borne fever reaction seemed to be preserved regularly up to 7 days when the sample had been stored at +4 C. Blood infected with J Puke strain once caused tick borne fever after storage for 14 days and once after 1 month of storage. In the latter case the incubation time was extended to seven days. With fresh blood the incubation period of J Puke strain in sheep usually varied between 2 and 4 days (18). On one occasion blood obtained from a reaction caused by J Puke strain proved negative after storage for 3 weeks. In summer 1966 a new *K. ingasniemi* strain was found to be infective after 14 days storage of the blood.

It was demonstrated that storage at -74 C regularly preserved the infectivity of blood obtained at the height of the reaction and frozen immediately for a period of several months at least. Table 2 lists the positive inoculations with blood stored in the frozen state.

These include samples each infected with one of seven different strains. The longest preservation recorded (J34-64 strain) was 8 months. An unnoticed fault in the deep freeze box which resulted in the thawing of samples precluded tests on preservation extending over 8 months. Inferior preservation in the frozen state was recorded when blood received from the field (6 cases) or blood stored for several days at +4 C was frozen. Only 2 blood samples from the field, one of which contained J34-64 strain and the other T1-65, were found to be infective after respectively 1 and 3 months storage at -74 C.

Both the storage of blood at +4 C and the freezing of blood and subsequent storage at -74 C tended to extend the incubation time beyond that recorded for fresh blood. Table 2 provides information in this regard on tests made with blood containing J34-64 strain.

TABLE 2

Length of Incubation Period in 19 Cattle and 5 Sheep Inoculated with J34-64 Strain and with no Previous Experience of Tick Borne Fever by Various Ways of Storage

Length of incubation period days	Fresh blood	Number of animals reacting to inoculation with					Total
		Blood stored at +4 C, days		Blood stored at -74 C, months			
		1-2	3-5	1-9	2-4	4-8	
4	2						2
5		1	1				2
6	1	1	1	2	1	1	7
7		1		7	2		10
8				1	1		2
9				1			1
10						1	1

Only inoculations with blood obtained at the height of the reaction are taken into consideration = includes one sheep

Tests of Cultivability and of Host Range of the Agent

Negative results were recorded in all attempts made to propagate the agent in conventional bacteriological media tissue culture embryonated hens eggs white mice guinea pigs pigs a horse and an elk calf No structures which simulated tick borne fever bodies were detected Only the two pigs exhibited a slight temperature rise 3 days after the inoculation even in their blood however no tick borne fever bodies were detected Subinoculations of pig blood were not carried out Subinoculation of the horse blood and the three subinoculations of the blood of the elk calf were all negative No cytopathic effect was observable in the tissue cultures and the calf inoculated with cells and medium from the calf kidney culture displayed no reaction

DISCUSSION

Some of the symptoms exhibited by affected cattle deserve special attention One interesting finding was the regular association of mild coughing with the condition of tick borne fever in animals which were inoculated with strains isolated from bovine field cases and kept under sufficiently close observation Hudson (11) and Overas & Hunshamar (13) recorded coughing in some of the affected cattle but considered that this might be merely coincidental The present results exclude coincidence as the explanation of this association and strongly suggest that coughing is part of the typical syndrome of bovine tick borne fever It has been previously reported that coughing was recorded in 41.9 per cent of 310 field cases of bovine tick borne fever (17) A reminder is necessary here that if close attention is not paid to the cough often silent it may easily escape notice

The pathogenesis of coughing in tick borne fever remains to be established It does not seem probable that it is directly effected by the tick borne fever agent British workers who made histological examinations of various organs of sheep and cattle killed during the disease attack found tick borne fever bodies in only blood granulocytes and monocytes (9-11) Foggie (3-4-5) has proved that an attack of ovine tick borne fever greatly diminished the resistance of sheep to staphylococci he considered that the impaired defence was attributable to the destruction of granulocytes by the tick borne fever agent Coughing in bovine tick borne fever coincides with the later part of the clinical infection at which stage the number of granulocytes is at its lowest It could be thought that the shortage of granulocytes might provide an opportunity for the normally nonpathogenic bacteria which inhabit the respiratory tract to cause a mild pathological reaction The development of discharges from the eyes and the nose might have a similar explanation

The findings in the present study support the impression derived from the results obtained in the field study (17) that Finnish strains

of bovine tick borne fever agent might in general be less apt to cause abortion than the Scottish strain described by Wilson *et al* (21)

In tick borne fever one frequent phenomenon is the appearance of a spontaneous relapse. In experimental cattle the interval between the end of the primary reaction and the appearance of the secondary reaction ranged from 3 to 8 days. However in the field study material the time interval that is the period between the observed commencement of both reactions varied from 11 to 18 days (17). Despite the difference in the ways of recording in the field material the actual interval was on the average definitely longer. Against this in three experimental sheep infected by J Puke strain intervals from 7 to 13 days were recorded a range which roughly corresponds to that in the bovine field material. As was mentioned above J Puke strain is highly virulent in sheep and the relapses stated were more severe than those recorded in the experimental cattle. Under field conditions the milder relapses of cattle might very well have gone unnoticed. The above findings may suggest that there exists a correlation between the length of the interval and the severity of both primary and secondary reactions. This may explain why only those relapses which appear after a relatively long interval are recorded under field conditions.

An intriguing question to which the answer can at present to a great extent be no more than a matter for conjecture is what makes the primary reaction subside and why this inhibition is occasionally so short lived that a secondary reaction will emerge? Such phenomena can hardly be explained in terms of antibody mediated immunity. It is more likely that the development of some kind of auto interference of limited duration is responsible for suppression and interruption of the reaction. The subject will be further discussed in the next paper of the series (18).

In principle the haematological findings correspond to those of Taylor, Holman & Gordon (16) in connection with ovine tick borne fever.

The successful subinoculation by 500 ml of blood obtained 9 days after the reaction and the simultaneous failure by 3 ml of blood emphasize that in all probability the conventional method of inoculating 3 to 6 ml was quite inadequate to reveal a low level carrier state and consequently no answer can be given to the question whether such a state was present in the investigated cattle. Splenectomy has been described as activating a latent tick borne infection in sheep (2). Splenectomies of calf 4 and sheep 2 were negative in this respect and may suggest that in these animals prior sterilization had taken place.

It seems apparent that strains of tick borne fever agent differ in their capability to induce a carrier state or at least in regard to the level of the state which they induce. The following facts appear to support this contention. In the present study J Puke strain which is highly virulent in sheep was recovered from the blood of sheep 2 about 1½ months after subsidence of the clinical reaction (the inoculum was

1 ml) In ovine tick borne fever caused by British strains which is usually a relatively severe affection a carrier state at least several months in duration was a frequent finding when subinoculations were made with a few ml of blood (2) On the other hand the Indian rather mild variant of the ovine tick borne fever agent was never recovered from the blood once more than 4 days had passed since the end of the clinical reaction (14) This information may suggest the existence of correlation between the virulence of the strain and the duration or level of the induced carrier stage As regards bovine tick borne fever the only mention of examination of the duration of the carrier state is that made by Hudson who found that blood obtained from a bovine 54 days after inoculation was negative (11) It cannot be known how far J34 64 strain typifies the behaviour of other Finnish bovine strains in the ability to remain latent but it is likely that they are inferior to British ovine strains in this respect

To judge from various reports the immunity caused by Finnish strains appears to be more solid and durable than that induced by most other strains investigated in this regard The only bovine strain on which data are available is that isolated by Hudson (11) Hudson reported that 11 out of 14 animals re inoculated six to twelve months after previous experience of the disease reacted like fully susceptible cattle He also mentioned that under natural conditions the disease is said to attack cattle quite commonly in successive lactation In Finland where the winter period during which cattle are housed and ticks are inactive is much longer than in England recurrence of the disease in later grazing seasons is nevertheless rare (17)

That strains may differ in the degree of protection they induce is also suggested by the conflicting findings made when different strains of ovine agent have been studied (2 10 15) A finding and clearly at variance with the Finnish experience was that the resistance to re infection by an ovine agent strain did not develop earlier than about 5 weeks after the primary infection (2)

The discovery that the agent can be preserved in the frozen state offered better opportunities for further studies It would not have been possible to make the immunological comparisons of several strains reported in the third paper of the series (19) if the only way to preserve strains had been continuous passage in experimental animals Recently Foggie Lumsden & McVeillage have also reported the successful preservation of ovine tick borne fever agent in the frozen state (8) These authors however used dimethyl sulphoxide or glycerol as preservative

The negative results of the attempts to propagate tick borne fever agent in systems other than those of cattle or sheep are complementary to previous findings by other workers (2 3 11) Although Foggie & Hood (7) succeeded in adapting the ovine agent to multiply in splenectomized guinea pigs and mice and subsequently also in non splen-

ectomized guinea pigs the virulence as regards these animals remained low. A rather strict species specificity seems to be a characteristic of tick borne fever agent.

By virtue of the related nature of the animal species concerned it was slightly surprising that the elk calf did not detectably react to any of the three strains inoculated. In Great Britain tick borne fever infection has been demonstrated in both red deer (5) and in fallow and roe deer (12). However it cannot be concluded on the basis of this limited experiment that elk is not susceptible to tick borne fever. Both the age (11) of the calf and the possible non virulence to elk of these specific strains might be sufficient to explain the negative result obtained here.

SUMMARY

The reaction to tick borne fever agent in 36 experimental cattle was characterized by moderate to high fever, slight to moderate depression and diminution of appetite. All the cattle inoculated with bovine strains and kept under close observation exhibited a mild cough. Some animals displayed further symptoms.

In rough coincidence with the fever reaction tick borne fever bodies were demonstrated in blood granulocytes and occasionally in monocytes as well. A short reappearance of bodies often associated with fever was observed in 10 out of 36 cattle.

Granulocytopenia was the most conspicuous of the hematological findings. The lowest counts were at about the end of the fever reaction.

No carrier state was apparent in 6 cattle and 2 sheep each infected with J34-64 strain when subinoculations were made with 3 to 6 ml of blood obtained between 3 days and 8½ months after the end of the clinical reaction. However an inoculum of 500 ml taken 9 days after the end of reaction caused tick borne fever. Additionally on the subinoculation of 1 ml of blood a splenectomized sheep was found to carry J-Puke strain after 1½ months.

In none of the 30 animals did challenge by a homologous strain within 3 months induce a detectable reaction. Two out of 5 animals which were challenged later on did react but only after a clearly prolonged incubation period.

On one occasion blood stored at +4° C for one month resulted in infection. Shell freezing of blood and subsequent storage at -74° C could preserve the infectivity for at least 8 months.

Attempts to propagate the agent in systems other than cattle and sheep were unsuccessful.

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SENSITIVITY OF VARIOUS SALMONELLA STRAINS TO FELIX 0-1 PHAGE

By

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Received 28 xii 60

The bacteriophage 0-1 of Felix & Collow (1943) has been found to react with a high degree of specificity on salmonella bacteria (Cherry *et al* 1954). When tested on more than 2 000 strains (Thal & Kallings 1955) 99.5 per cent of the salmonella cultures were lysed as opposed to only 0.3 per cent of the non salmonella members of the Enterobacteriaceae family. Due to this specificity the 0-1 phage has been used as an additional tool for the routine identification of salmonella bacteria in Sweden. The usefulness of the 0-1 test has been found to vary a great deal with the technique applied and the type of material tested. The effective multiplicity of infection for various salmonella strains is described in the present paper. The results are analyzed in connection with the ability or inability of the bacteria to support virus growth.

MATERIALS AND METHODS

Bacteria: The strains were isolated from clinical specimens or originated from cultures submitted to the National Bacteriological Laboratory for typing. When not tested directly they were stored in sealed agar stab tubes at room temperature. If not otherwise stated cultures grown in broth for four hours at 37 °C were used for experiments. The bacterial concentration was estimated by colony counts or in some cases by the aid of a nephelometer standardized by colony counting.

Phage: Felix 0-1 phage was propagated on strain B76, a paratyphi B phage type 1 by the agar layer method. Sterile lysates filtered through Jena sintered glass filters and stored undiluted at +4 °C were used as stock suspensions. The number of infectious phage particles was estimated by the standard agar layer method. 10 ml phage dilution was pipetted into a tube containing 8 ml 0.6 per cent melted agar at 45 °C to which 0.1 ml cultures of B76 had just been added. After mixing, 2 ml were poured over an agar plate containing 10 per cent agar medium. The plaques were counted on the following day after incubation at 22 °C overnight. The absolute efficiency of plating was unknown but with regard to the high plaque counts (10⁷) it was assumed that the plaque count corresponded closely to the true number of infectious particles.

As a standard method for spot tests a loopful of bacterial broth culture was inoculated over a circular area (17 mm diameter) on 10 per cent agar plates by a platinum loop of 2 mm interior diameter giving an average drop volume of 0.005 ml. Using the same kind of standardized loop the phage dilution was spotted on the inoculated areas after they had dried. The phage drop covered a circular area of

Preliminary report given 1961 to the Medical Microbiology Division of the Swedish Medical Society (Kallings 1962).

With the technical assistance of Mrs. Haul Lindberg and Mr. Cerd Lundström

about 9 mm diameter. The readings were taken after five hours incubation at 37 °C. The multiplicity of infection obtained with this technique is calculated in Table 1.

The one step growth curve on the propagating strain of phage 0-1 had been determined by repeated experiments in order to learn some of the characteristics of the phage. At a virus concentration of 0.2 phage particles per bacterium (3×10^8 viable cells per ml) on an average 40 per cent of the phage particles were adsorbed to the host cells within 10 minutes at 37 °C. Phages were first liberated 75 minutes after infection and the titer increased over a period of 10 minutes. The burst size was about 220 infectious particles per bacterium.

Media. The same Difco nutrient agar and broth as described in a previous paper (Hallings 1961) was used if not otherwise stated.

Diluents. Nutrient broth (Difco) or phosphate buffered saline pH 7.4.

TABLE 1

Calculated Multiplicity of Infection with the Standard Spot Test Technique

	Titer of suspension	Spot diam mm	Particles or cells per loop	mm
Phage	1.9×10^{11}	9	1.7×10^3	9.7×10
Bacterium	1.0×10^8	17	8.8×10	3.9×10^8

Resulting phage to bacterium ratio 6.9×10^3

Figures taken from the experiments summarized in Table 3

RESULTS

Routine use of the 0-1 phage on clinical and culture material from different sources. The results from routinely performed 0-1 tests on consecutive isolations from faecal specimens or on cultures submitted to the laboratory for identification were compared over a period of time chosen at random. The comparison was comprised of (group I) 521 salmonella and 695 non salmonella strains tested at a laboratory section for the routine isolation of salmonella organisms from clinical specimens (group II) 215 salmonella and 52 non salmonella cultures submitted to the National Salmonella Center and (group III) 2 200 *S. paratyphi* II and *S. typhi* murium cultures examined at a laboratory section for enteric phage typing. The salmonella bacteria of the first and second group consisted of a variety of serological types.

The 0-1 tests in the first two groups were performed in the same way: an isolated colony from a culture was streaked on an Endo's agar plate (about 1.3 per cent agar) with a platinum loop and a loopful of undiluted phage suspension containing 10^{-10} plaque forming units (pfu) per ml was spotted on a closely streaked area of the plate. The results were read after overnight incubation at 37 °C. In the third group undiluted phage suspension of the same strength as above was spotted on peptone agar plates (1.0 per cent agar) previously inoculated with overnight or actively growing broth cultures to give areas of confluent bacterial growth. The results were read after five hours.

A reaction characterized by no bacterial growth or less commonly by secondary growth within the area of the phage drop was classified as

positive Reactions with semi confluent lysis or isolated plaques were not found when undiluted phage suspensions were used. An unbroken bacterial lawn, sometimes with a slight thinning of the growth within the area of the phage drop, was classified as a negative reaction.

In the first group of strains chosen for the comparison, a positive phage reaction was shown by 98.8 per cent of the *Salmonella* (Table 2) as opposed to 1.2 per cent by the non *Salmonella*. The corresponding percentages for the cultures of group II were 75.4 and 5.8. In group III 99.9 per cent were 0-1 positive. The agreement between the results of the serological typing and of the 0-1 test thus was satisfactory for the strains examined at the routine diagnostic and phage typing sections but poor for the strains examined at the *Salmonella* Center.

TABLE 2

Results from Routine 0-1 Phage Tests on Materials from Three Different Sources

Sources of cultures	Number of cultures					
	Total	Salm	Pos phage react	%	Non Salm	Pos phage react
I Diagnostic routine lab	1 216	521	515	98.8	695	8
II <i>Salmonella</i> Center	267	215	162	75.4	52	3
III Phage typing ref. lab	2 260	2 250	2 258	99.9	0	-

Interic gram negative rods

TABLE 3

Comparison of Results Obtained with Different Techniques and Phage Concentrations on Material from Group II in Table 2

Technique and phage conc	Number of cultures					
	Salm	Pos phage react	%	Non Salm	Pos phage react	%
Endo's agar						
Phage titer = 10^9	215	162	75.4	52	3	5.8
Phage agar						
Phage titer = $\times 10^{11}$	215	214	99.5	52		9.8

These figures for group II in Table 2 are included for comparison.

Importance of phage concentration and test technique. The strains routinely tested at the *Salmonella* Center were retested using the media and spot technique described under "Materials and Methods." The titer of the phage suspension used was now considerably higher, about 2×10^{11} pfu/ml. The multiplicity of infection in the spots was about 7 000.

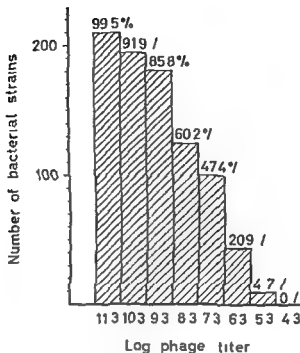


Fig 1

Spot test on 211 *Salmonella* strains

Distribution of positive reactions with various concentrations of phage 0-1

(Table 1) Only one of the salmonella strains (101/60 *S. infantis*) was found to be 0-1 negative (Table 3). On the other hand the percentage of positive reactions among the non salmonellas had increased from 5.8 to 9.6.

The plaque size is known to be inversely related to the agar concentration. The different agar concentrations used for the Endo's agar plates at the Salmonella Center and for the plates employed for the specially adopted technique might be partly responsible for the difference in the results shown in Table 3. Thus it was found that five times as high a phage concentration was necessary to produce the same reaction on Endo's medium containing 1.6 per cent agar (plaque diameter about 0.4 mm) as on 1.0 per cent nutrient agar or Endo's agar (plaque diameter about 0.8 mm).

To study the sensitivity of the strains to various concentrations of 0-1 phage 211 of the originally available 215 strains sent in to the Salmonella Center were examined by spot tests of ten fold dilutions of the phage suspension. As demonstrated in Fig 1 the sensitivity to the phage was found to vary greatly. At a phage concentration of 2×10^{10} pfu/ml 16 strains were negative in addition to the one negative at a ten times higher concentration. Each ten fold dilution of phage caused a stepwise loss of the number of strains showing a positive reaction. The most sensitive strains were lysed confluent by a phage dilution containing 2×10^5 pfu/ml. At high dilutions of the phage sus-

pehension isolated plaques of about the same size (about 0.8 mm) as produced in the propagating B76 strain were observed in 107 (51 per cent) of the strains (here designated as strains of *reaction type I*). By counting the number of isolated plaques it was found that 25 strains had about the same high sensitivity to the phage as B76.

Irrespective of the phage dilutions no isolated plaques visible to the naked eye were found in the remaining 49 per cent of the strains (strains of *reaction type II*). All these strains were sensitive only to high phage concentrations, the most sensitive giving full positive reactions at a phage titer of 2×10^8 . The reaction in some strains changed from positive to completely negative by one dilution step; the reaction in others changed more gradually on dilution of the phage by exhibiting a reaction characterized by an irregular inhibition of the bacterial growth within the area of the phage drop.

According to the findings of Cherry *et al.* (1954) a high percentage of resistance to phage 0-1 was found among strains classified in group E of the Kauffmann-White scheme. In the present material the difference in serological characteristics between the strains lysed by plaques and those affected differently is analyzed in Table 4. In all the strains represented 57 different serotypes belonging to 15 O-groups or subgroups, the most common types being *S. typhi* murium and *S. montevideo* with 34 and 53 strains respectively. As seen in the table both reaction types occurred in cultures belonging to 7 of the 9 groups represented by more than one strain. Both reaction types were found also

TABLE 4
Distribution of Serological Groups of Salmonella Strains of Varying Sensitivity to Phage 0-1

Serological groups	Number of strains belonging to		Total number of strains
	<i>reaction type I</i>	<i>reaction type II</i>	
A	7		7
B	30	34	64
C 1	42	38	80
C 2	8		8
C 3		1	1
D 1	11	10	16
D 2	1	1	2
E 1	5	13	18
E 4	1	5	6
F	3	1	4
H	1		1
I	1		1
L	1		1
M	1		1
N		1	1
Total	107	104	211

According to the Kauffmann-White scheme

among most of the individual sero types. However all 8 strains of *S. enteritidis* belonged to reaction type II. At least 4 of them were epidemiologically unrelated.

Plaque formation in agar layer. The inability of the virus to produce plaques observed in spot tests on the strains influenced only at high multiplicities was further confirmed by experiments in which broth cultures grown for four hours were mixed with equal volumes of 0-1 phage diluted in ten fold steps to 10^{-8} . The titer of the stock suspension was 10^{10} pfu/ml. Six cultures of reaction type II were tested (five sero types: *S. typhi*, *murium*, *S. montevideo*, *S. enteritidis*, *S. cholerae* *suis* and *S. bareilly*). The mixture of phages and bacteria were plated by the standard agar layer method. No plaques were observed. When undiluted phage suspension or a dilution 1:10 was used only scanty or no bacterial growth was observed in contrast to the abundant growth at higher phage dilutions. No plaque formation was revealed even when two fold dilution steps were made in the range between the concentrations lysing the bacteria or inhibiting the bacterial growth and the concentrations of phage which had no obvious influence on the bacteria.

Effect of a phage free lysate and a purified phage preparation. A high titer phage suspension was passed through a membrane filter (Gottingen ultrafine porosity 35-100 μ). The undiluted phage free filtrate was spotted on cultures on the propagating B76 strains and on five strains of reaction type II. The spot test was negative for all strains including the very sensitive B76. A phage preparation purified by a two phase system (dextran methylcellulose, Albertsson 1960) and containing 5×10^{11} pfu/ml was found to cause positive reactions in all six of these strains.

Bacterial survival and growth after phage adsorption. A series of experiments indicated that the 0-1 phage was adsorbed also to salmonella bacteria of reaction type II even to the only spot test negative salmonella strain found in the present study (101/60 *S. infantis*). In some of these experiments phage adsorption was followed for periods up to 21 hours at 37°C by assay of the unadsorbed phage. The finding of continuously diminishing number of free particles verified the inability of these strains to propagate phages.

TABLE 5
Per Cent Surviving Bacteria after Adsorption of Phage 0-1

Strain	Per cent survivors
B76 (<i>S. ba. typhi</i> B)	1
87/61 (<i>S. bareilly</i>)	44
88/61 (<i>S. muenster</i>)	57
98/61 (<i>S. muenster</i>)	57
183/61 (<i>S. typhi murium</i>)	3
2132/61 (<i>S. enteritidis</i>)	41
101/60 (<i>S. infantis</i>)	19

Means of duplicate experiments

Adsorption mixtures. Equal volumes of a pre-armed 0-1 suspension (6×10^{10} pfu/ml) + a 4 hour *Salmonella* broth culture (2.5×10^8 cells/ml). Incubated 10 minutes at 37°C and diluted 10^{-5} . Unadsorbed phages were neutralized by adding 0.2 ml of the dilution to 0.8 ml anti-0-1 serum (1:50) and incubating at 37°C for 5 minutes. 0.5 ml were then spread on an agar plate and colonies counted after incubation at 37°C.

Control mixtures. Equal volumes of the *Salmonella* broth culture and nutrient broth treated in the same ways as the adsorption mixtures.

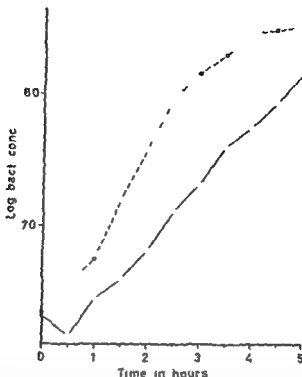


Fig. 2

Growth curve of an O-2 negative *Salmonella* culture in the presence of the O-1 phage. Phage O-1 + bacterium mixture (x — x) One part washed cells from an overnight broth culture of 101/60 *S. infantis* suspended in broth (2×10^8 cells/ml) + nine parts of a broth dilution of purified phage O-1 (8×10^{10} particles/ml) in incubation in a waterbath at 37°C. At each time interval samples of 0.1 ml were appropriately diluted. 0.5 ml of the dilutions were mixed with agar and poured on agar plates for colony counting after incubation at 37°C overnight.

Control mixture (o — o) One part bacterium suspension + nine parts of a broth dilution of purified coli phage T₂ treated in the same way as above.

The relative resistance of strain 101/60 and five strains of reaction type II compared to strain B76 of reaction type I was demonstrated in an experiment where the bacteria were infected with a multiplicity of 240.

As seen in Table 5 only one per cent of the B76 survived after the adsorption time and phage input employed whereas the survival of the bacteria belonging to reaction type II was considerably higher 29 to 57 per cent.

Although the spot test on strain 101/60 was registered as negative (see Table 3) it was found that the bacterial growth after five hours incubation was thinner over the area corresponding to the phage drop than over the surrounding area. Since previous results suggested that the phage adsorbed to this bacteria which resulted in a decrease in the number of viable organisms (Table 5) the bacterial growth in the presence of excess phage (40 000 phages per bacterium) was studied further for the first five hours of incubation (Fig. 2).

An initial drop in the cell count was noted but this decrease was followed by a phase of rapid growth. During the period studied the concentration of living cells in the phage bacterium mixture was at all times lower than the concentration in the control culture. Thus it was found that the only salmonella strain from the Salmonella Center remaining negative in the spot tests in fact was affected also by the 0-1 phage.

DISCUSSION

The present investigation shows that the concentration of the phage is an important variable if the 0-1 phage is to be used as an additional aid in routine salmonella identification. To obtain almost 100 per cent positive reactions on salmonella strains a high phage input and a low agar concentration are necessary. However these measures also will increase the number of positive reactions in cultures not belonging to the salmonella genus. Furthermore the agar concentration in e.g. Endo's agar medium cannot be decreased to a level ideal for plaque formation if the bacterial colonies are to be used for slide agglutination tests. In such a case the agar concentration has to be high enough to allow the formation of sufficient O-antigens in relation to H-antigens. When as a matter of convenience the 0-1 test is performed on the same agar plate as used for the routine morphological and serological identification of salmonella the choice of agar concentration has to be a compromise.

The discrepancy between the occurrence of 0-1 positive non salmonella strains in the material from the different sources 12 against 58-96 per cent may be due to chance considering the small number of strains from the Salmonella Center but may also be due to some form of selection in this group. Since the 0-1 phage is used as an additional diagnostic test at many laboratories certain serologically negative or doubtful cultures are forwarded to the Salmonella Center for further examination mainly because they were found to be 0-1 positive. Some salmonella strains are indeed traced down in this way but many cultures prove not to be Salmonella. However the occurrence of 0-1 positive cultures not classified as Salmonella has not been reported as a serious disadvantage from other Swedish laboratories. The information collected about the test in the routine diagnosis of salmonella organisms seems to justify a continuation of its use as an additional tool provided that a standardized technique is employed.

The existence of a phage capable of adsorbing to almost all salmonella bacteria suggests the presence of a common structure in the cell walls and provides a tool for comparative investigations of the cell wall composition by studies of the receptor (Lindberg 1967).

In about half the number of the salmonella strains from the selected material investigated phage adsorption killed or inhibited the growth of the cells without signs of virus synthesis whereas in the other strains phage adsorption was found to result in an ordinary lytic cycle with synthesis of new virus particles. Investigations on the cause of this

difference (Kallings & Lindberg 1967) indicate that the relative resistance to phage 0-1 may be due to the presence of prophage in the bacteria.

SUMMARY

A comparison has been made between the results from the routine use of the 0-1 phage spot test on about 3 000 salmonella strains and about 700 other enterobacterial strains at three laboratory sections working with cultures from different sources and using different techniques (routine salmonella diagnosis, salmonella reference identification and enteric phage typing). In the order of the sections listed, positive phage reactions were obtained in 98.8, 75.4 and 99.9 per cent of the serologically verified salmonella strains.

When the strain material showing only 75.4 per cent positive reactions was retested with a high titer phage preparation and a special standardized technique 99.5 per cent positive reactions were obtained. It was found that plaques were produced in 51 per cent of these strains which were lysed by low phage inputs. No plaques were observed in the remaining 49 per cent of the strains. They were only affected by high multiplicities of infection but did not support phage propagation.

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RESISTANCE TO FELIX 0-1 PHAGE IN SALMONELLA BACTERIA

By

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The bacteriophage 0-1 capable of acting on most salmonella bacteria has been found to separate the salmonella strains into two groups (1) strains lysed by low phage concentrations and supporting the propagation of new particles and (2) strains influenced only at high phage concentrations but unable to produce phages (Kallings 1962 1967) The purpose of the present paper was to study the cause of the different action of the phage As the 0-1 phage particles were found to be adsorbed by the salmonella organisms influenced only at high phage concentrations the inability to produce 0-1 phages was assumed to be due to the presence of prophage In the present paper experiments are reported concerning the isolation of temperate phages and attempts to transform sensitive strains to resistant ones by means of these phages

MATERIALS AND METHODS

Bacteria Salmonella strains were identified by common biochemical and serological procedures All strains were isolated from clinical specimens and stored less than two years in sealed agar stab cultures Unless otherwise stated the bacteria were grown in broth for four hours at 37 °C

Phage 0-1 phage (Felix & Callow 1943) was propagated on strain B 76 (*S. paratyphi B*) by means of the agar layer method (Adams 1959) Lysates sterilized by filtration through Jena glass filters and stored undiluted at +4 °C were used as stock suspensions

Temperate phages isolated from lysogenic cultures were propagated on *S. typhimurium* strains by the same method and stored under same conditions

Media The same Difco nutrient agar and broth described in a previous paper (Kallings 1961) was used In addition the synthetic medium M 9 (Adams 1959) was used

Diluents Nutrient broth or phosphate buffered saline pH 7.4 was used as diluents

Induction The bacteria were adapted to grow in M 9 medium at 37 °C Subcultures in M 9 were grown at 37 °C for four hours A shallow layer of the culture in a Petri dish was exposed for 10 seconds to ultraviolet light (2537 Å) at a distance of 50 cm with continuous shaking Afterwards the bacteria were incubated at 37 °C for 30 minutes and centrifuged at 2000 g for 20 min

Preliminary report given to the Medical Microbiology Division of the Swedish Medical Society 1967

With the technical assistance of Mrs Maud Linberg and Mrs Gerd Lundström

Phage assay The phage concentration was measured by plaque counting or the spot technique described previously (Kollings 1967)

Antiphage sera Hyperimmune sera against phage 0-1 and phages 105 35 27 88/61 and 250/61 were produced by injecting rabbits subcutaneously with stock phage suspensions. The time schedule was 2 injections per week of 5 ml each for 4 weeks followed by 5 ml twice after another two weeks. The sera were inactivated at 56 °C for 30 min.

Neutralization test A mixture of 0.5 ml phage dilution (containing about 1.0×10^5 pfu/ml) and 0.5 ml diluted antisera were incubated at 37 °C for 15 minutes in a water bath. From this mixture 0.5 ml were mixed with 4.0 ml soft melted agar at 45 °C plus 0.5 ml of a bacterial culture. 2 ml were plated on an agar base and incubated at 37 °C overnight. The degree of neutralization was determined by comparing the number of plaques formed with that of controls (phage + broth).

EXPERIMENTAL

Isolation of Phage 105 from a Lysogenic Salmonella Strain

Twenty *Salmonella typhimurium* cultures of different phage and fermentation types were tested for sensitivity to ten fold dilution steps of the 0-1 phage by the spot technique. Readings were carried out after five hours incubation at 37 °C. Reactions without any evidence of phage multiplication and an unbroken bacterial lawn were classified as *negative*. Reactions characterized by a clearly lysed area (i.e. no bacterial growth) semiconfluent lysis or isolated plaques within the area of the phage drop were considered as *positive*.

Eighteen cultures were lysed supporting the propagation of new particles at low multiplicities of infection. The two remaining cultures gave negative reactions at low multiplicities. Although influenced by high phage concentrations they were unable to maintain the propagation of the 0-1 phage. One of these two cultures (S 105) was chosen for an attempt to isolate a temperate phage responsible for the resistance against phage 0-1.

After induction of strain 105 the supernatant was tested against the 20 *S. typhimurium* strains by the spot technique. An isolated plaque on one (S 204) of the strains lysed was selected. By repeated isolations and subcultures of single plaques on the strains S 204 a phage suspension labelled 105 with a titer of about 1.0×10^5 pfu/ml was prepared.

The phage was again tested against the 20 *S. typhimurium* strains as above. All strains but strain 105 were now lysed.

No contaminating phages originating from the propagating strain (S 204) or phage 105 could be demonstrated when the strain was induced and the supernatants tested against the 20 indicator cultures.

Conversion to 0-1 Resistance by the Phage 105

Phage 105 was spotted on the 18 strains lysed by phage 0-1. After prolonged incubation isolated colonies growing within the area originally lysed confluentlly were picked up and identified as *S. typhimurium*. The bacteria were then tested for sensitivity to phage 0-1 by spot technique. Resistant bacteria were found in 8 of the 18 cultures.

These bacteria were affected only at high multiplicities of infection (without plaque formation)

To check if the 8 *S. typhi murium* cultures were lysogenized by phage 105 the cultures were induced with ultraviolet light. The supernatants were tested against the 20 *S. typhi murium* indicator strains by the spot test. Strong reactions (confluent or semiconfluent lysis) appeared on all strains with the exception of strain 105.

The phages obtained by induction of the 8 converted cultures were neutralized by hyperimmune serum against phage 105 (Table 1).

TABLE 1
Neutralization of Phages Isolated from Lysogenic *Salmonella* Cultures
by Antiserum against Phage 105

Phages or induced culture supernatant	Antiserum	Per cent neutralized
238	105	98.4
237	105	98.0
207	105	100
181	105	97.4
100	105	99.1
99	105	99.9
7	105	99.1
561	105	99.5
105	105	100

Dilution of antiserum against phage 105: 1:250

The neutralization of phages derived from lysogenic cultures ranged between 98 and 100 per cent. With two exceptions unneutralized phage particles gave rise to plaques with the same morphology as those caused by phage 105. The two exceptions were from the supernatants of the cultures 237 and 238 which in addition to plaques with the characteristic size and shape of phage 105 yielded larger plaques with a more pronounced turbid center. The unneutralized phage particles could be either phage 105 which had not been neutralized or other phages released from the various *S. typhi murium* cultures during this last induction. To investigate this possibility the two different plaque types were cut out, propagated and used in a new neutralization test with hyperimmune serum against phage 105.

The turbid plaques from supernatants 237 and 238 yielded phages which were not inactivated by hyperimmune serum against phage 105.

Inability to Propagate Phage 0-1 on the Lysogenized Cultures

0-1 phage was used to infect actively growing cultures of the 8 lysogenized *S. typhi murium* strains at a concentration of phage which used to cause semiconfluent lysis on the unconverted bacteria. The experiment was designed to see if phage 0-1 could multiply within these bacteria.

Table II shows that *Salmonella* strain 105 which yielded the temperate phage 105 was unable to maintain the propagation of phage 0-1. As a comparison strain 201 the propagating strain of phage 105 gave rise to about a 400 fold increase in phage 0-1 titer. The phage was unable to

multiply also on the lysogenized cultures 99 and 561 and gave a small rise in titer on the other 6 cultures

TABLE 2
Propagation of Phage 0-1 on the Lysogenized Cultures

Strain	Original 0-1 titer (pfu/ml)	Resulting 0-1 titer (pfu/ml)	Difference in multiples
10a	1.0×10^8	4.6×10^8	- 2
238	3.4×10^8	1.5×10^9	+ 4
237	3.4×10^8	1.0×10^9	+ 4
207	3.4×10^8	4.5×10^8	+14
131	3.4×10^8	2.6×10^9	+ 8
100	3.4×10^8	1.2×10^9	+ 4
99	3.4×10^8	3.0×10^8	± 0
7	3.4×10^8	1.2×10^9	+ 4
561	3.4×10^8	2.7×10^8	- 1
204	1.1×10^8	3.9×10^9	+390

The slight amount of phage 0-1 propagation in some of the lysogenized strains may be due to a small portion of the bacterial population which do not contain prophage. Phage 0-1 propagated on the unconverted indicator strains gave 30-1300 folded rises in titers.

Isolation of other Temperate Phages Causing Resistance to Phage 0-1

To investigate the frequency of lysogeny causing 0-1 resistance 34 *Salmonella* strains (belonging to 10 serotypes) lysed only by high 0-1 phage concentrations without phage multiplication were induced with ultraviolet light and treated as above. The supernatants were plated on the 20 *S. typhi murium* cultures earlier used as indicator strains by the spot technique to determine the presence of phages. Four strains were found to harbour phages which produced large turbid plaques which were different from the phage 10a plaques. Two of these phages were capable of converting three of the indicator strains to 0-1 resistance. The strains harbouring phages with converting ability belonged to serogroup F: *S. veyle* (23a/61) and *S. muenster* (88/61). The phage from *S. muenster* was capable of converting two strains while the phage from *S. veyle* converted one. In this experiment nonconverting phages were obtained from a *S. typhi murium* (27/61) serogroup B and a *S. montevideo* (2a0/61) serogroup C.

The three converted cultures were induced in order to investigate if they were lysogenized with phage 235/61 or 88/61. Supernatants were tested in a spot test against the 20 *S. typhi murium* indicator strains. Positive reactions mostly less than semiconfluent lysis appeared on 18 strains this time including strain 105. Hyperimmune serum prepared against the two phages neutralized between 94 and 100 per cent of the phages in the supernatants.

Serological Relationships between Phage 0-1 Resistance Converting and Non Converting Phages

The converting phages 105 235/61 88/61 nonconverting phages 27/61 250/61 and phage 0-1 were compared in a neutralization test

TABLE 3
*Cross reactions in a Neutralization Test between Phage 0-1
Immunity Converting and Non Converting Phages*

Anti Sera	Serum dilution	0-1 phage	Converting phages			Nonconverting	
			105	235/61	88/61	27/61	250/61
0-1	1 1000	+++	—	—	—	—	—
105	1 250	—	+++	—	+	—	+
235/61	1 100	—	+	++	+	+	+
88/61	1 100	—	—	++	+++	++	+++
27/61	1 50	—	—	++	+++	+++	—
250/61	1 50	—	—	+++	+++	+++	+++

+++ complete neutralization
++ 50-99 per cent neutralization
+ 25-49 per cent neutralization
— < 25 per cent neutralization

Except for phage 0-1 anti-0-1 serum did not inactivate any of the investigated phages. Serum against phage 105 gave weak cross reactions with phages 88/61 and 250/61 while serum against phage 235/61 gave a weak reaction with phage 105. Cross reactions were frequently detected within the temperate phages 235/61 88/61 27/61 and 250/61.

CONCLUSION

As has been shown in a previous paper (Hallings 1967) only about 50 per cent of salmonella strains belonging to different serotypes were able to support phage 0-1 propagation. However the majority of the other 50 per cent were found to adsorb the 0-1 phage although the virus did not multiply. In the present paper this inability of phage multiplication in spite of adsorption was shown to be due to the presence of prophage in some strains isolated from clinical cases of salmonellosis. The temperate phages were isolated and used to convert 0-1 sensitive salmonella strains into 0-1 resistant ones.

Temperate phages isolated from one salmonella serogroup (E1) were able to convert bacteria of another serogroup (B) to 0-1 resistance. The converting phages isolated from different sources were not identical as judged by plaque morphology and serology. However a certain serological relationship was demonstrable. The converting phages were as well serologically related to non converting phage isolated from other lysogenic salmonella cultures. None of the phage isolated whether con

verting or not was serologically related to the 0-1 phage. The broad antigenic relationship of various phages isolated from lysogenic salmonella cultures is notable.

SUMMARY

Although many salmonella cultures were lysed by high concentrations of 0-1 phage they could not support virus multiplication. This was shown to be due to the presence of prophage. Resistant bacteria were induced to release temperate phages capable of converting 0-1 susceptible salmonella strains to resistant ones.

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AN ANAEROBIC ORAL FILAMENTOUS ORGANISM POSSIBLY RELATED TO *LEPTOTRICHIA BUCCALIS*

2 Composition of Cell Walls

By

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In a previous paper some morphological physiological and serological data of an anaerobic oral filamentous organism were described (9). The organism had some properties in common with *Leptotrichia buccalis* but differed from that species in showing a heterofermentative type of sugar metabolism. In addition little or no serological cross reactivity was found between the filamentous organism and typical *L. buccalis* strains.

The present paper deals with the chemical composition of cell walls from the atypical filamentous organism and from *L. buccalis*.

METHODS

Preparation of Cell Walls

Cell walls were prepared from the following filamentous strains 1^o L23 L44 L49 and L52 and from the *L. buccalis* strains L11 and L66. The strains which were also included in the previous study (9) were grown in nutrient broth enriched with 0.1 per cent cysteine hydrochloride 0.3 per cent yeast extract (Difco) 1 per cent glucose and 11 per cent of human ascitic fluid. After 2 to 4 days incubation at 37 °C, the bacteria were harvested by centrifugation washed in saline and kept frozen until used.

The procedures employed for preparation of the walls were essentially as described by Yoshida et al. (15). The density gradient centrifugation was carried out at 900 × G for 1 hr. The washed walls were stored in the freeze dried state.

Trypsin digestion of the prepared walls was performed with crystalline trypsin (Trypsin Novo) freshly prepared in 0.05 M tris buffer of pH 7.8 using 0.1 mg trypsin per mg walls. The digestion was carried out at 37 °C for 6 hrs.

Paper Chromatography

Hydrolysis was performed in sealed tubes in 0.1 N HCl and 3 N HCl for 7 and 3 hrs at 100 °C, respectively and in 6 N HCl for 18 hrs at 100 °C. The dried materials were made up in distilled water and subjected to circular chromatography using Whatman paper No. 1.

Developing Solvents (A) n-butanol acetic acid water (4:1:1) and (B) phenol water (4:1 w/v) for amino acids and amino sugars (C) 1-butanol pyridine water (6:4:3) for neutral sugars (D) n-propanol ammonia (d 0.91) water (6:3:1) for

sugar alcohols and (E) iso propanol 2 N hydrochloric acid (65:35) for purine and pyrimidine derivatives.

Spray reagents: Amino acids were detected with 0.4 per cent ninhydrin in acetone containing 2 per cent acetic acid and aldoses with aniline hydrogen phthalate in water saturated n butanol. The Fison Morgan reagent as modified by Lartridge was used for detection of amino sugars. Sugar alcohols were examined for by sodium periodate benzidine and proline and hydroxyproline by 5 per cent isatine in water saturated n butanol containing 4 per cent acetic acid.

Quantitative Analyses

The amino acid content in 0.1 N HCl hydrolysates of undigested and trypsin treated walls was determined by the ninhydrin method essentially as described in (10). A solution of equal parts of glutamic acid, alanine and diaminopimelic acid was used as standard.

Hexosamines were determined by the method of Randle & Morgan (11).

Estimation of neutral sugars was performed with α naphthol as described in (10). Glucose was used as standard.

The sulphuric acid cysteine reaction of Dische (9) was used for the detection and quantitative determination of heptose. Standard samples of different aldoheptoses were kindly supplied by Dr A. K. Richtmyer, National Institutes of Health, Bethesda, Md.

The sulphuric acid cysteine reaction was also used for determination of rhamnose (3).

Lipid was estimated by determination of lipid ester groupings as described by Snyder & Stephens (14).

EXPERIMENTAL PROCEDURES AND RESULTS

Almost complete disruption as judged by Gram staining, was obtained by passing the frozen bacteria twice through the N press (see 15). The disintegrated material tended to clump even after removal of the protoplasm fraction and repeated washings. The cell wall layer in the middle of the sucrose gradient tube was therefore very diffuse and considerable amounts of walls were lost in the sediment which contained the few whole cells.

The freeze dried wall preparations varied in colour from white to light grey. The preparations were free from nucleic acids as no purine or pyrimidine bases were found when 0.1 N hydrochloric acid hydrolysates were examined by paper chromatography.

Digestion with trypsin was carried out on wall preparations from strains L14, L49 and L11. Following this treatment the preparations lost two thirds in weight, varying somewhat from one batch to another. The freeze dried trypsin digested walls were white and contrary to the undigested walls, homogeneous suspensions were easily obtained with water or buffers.

Paper Chromatography

The amino acids and neutral sugars found in hydrolysates of undigested and trypsin digested walls are listed in Tables 1 and 2 respectively. While the filamentous organism and *I. buccalis* contained the same amino acids in their walls, the filamentous organism differed clearly from *I. buccalis* with respect to cell wall sugars.

TABLE 1

Amino Acids in 6 N Hydrochloric Acid Hydrolysates of Cell Walls from the Filamentous Organism (3 Strains) and L. buccalis (2 Strains)

	Undigested walls	Trypsin digested walls
Alanine	++	+++
Glutamic acid	++	+++
Diaminopimelic acid	++	+++
Glycine	++	++
Lysine	++	+
Serine	++	+
Valine	++	+
Leucine	++	+
Threonine	++	+
Aspartic acid	++	—
Proline	+	—
Methionine	+	—
Arginine	+	—

In addition traces of a slow moving component were seen in hydrolysates of *L. buccalis* run in system (B)

+++ ++ + Relative amounts judged by visual comparison of the colour intensity on ninhydrin treated chromatograms

— Not detected

TABLE 2

Neutral Sugars in 3 N Hydrochloric Acid Hydrolysates of Cell Walls from the Filamentous Organism (5 Strains) and L. buccalis (2 Strains)

	Filamentous organism		<i>L. buccalis</i>	
	Undigested	Trypsin digested	Undigested	Trypsin digested
Glucose	+++	+++	+++	+++
Galactose	—	—	+	+
Rhamnose	++	++	—	—
Heptose	++	—	—	—

In addition traces of arabinose were found in undigested walls from *L. buccalis*
+++ ++ + Relative amounts judged by visual comparison of the colour intensity of aniline hydrogen phthalate treated chromatograms

— Not detected

Glucosamine and muramic acid were found in undigested and trypsin treated walls from all strains. Sugar alcohols were not detected in 3 N hydrochloric acid hydrolysates of undigested walls.

Nature and Location of the Heptose in the Cell Wall

The sugar listed as heptose in Table 2 gave the same red brown colour with aniline hydrogen phthalate as standard samples of aldo heptoses. It moved like D glycerol D galactohexose in the solvent systems (B) and (C).

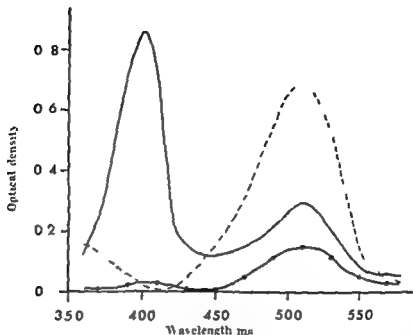


Fig. 1

Dische reaction spectra given by ——— 500 μ g cell walls from the filamentous organism strain L-44 ●—● the isolated heptose read against a paper blank from the same chromatogram - - - - 100 μ g Π glycero Π galacto heptose

In order to confirm this chromatographic finding the assumed aldo heptose was isolated and subjected to the sulphuric acid cysteine reaction of Dische. The isolation was performed in the following way: an evaporated 3 N hydrochloric acid wall hydrolysate (strain I-44) was made up in distilled water and passed through a column of Amberlite IR-120 in the hydrogen form. While the amino acids and amino sugars were retained by the resin the neutral sugars went straight through the column. The fractions containing the sugars were evaporated *in vacuo* or dissolved in a small volume of distilled water and subjected to paper chromatography in solvent system (C). After having traced the different sugars by treating small radiate strips of the dried chromatogram with aniline hydrogen phthalate the heptose was eluted from the paper with distilled water.

The absorption spectra of the chromogens produced in the Dische reaction by the isolated heptose and a sample of undigested walls from strain I-44 are shown in Fig. 1. The high optical density at 400 mμ produced by the wall sample is due to rhamnose and glucose which produce absorption maxima at 400 and 410 mμ respectively.

The aldohexose was only found in minimal amounts in hydrolysates of the protoplasm fraction of the bacterial cells (the supernatant fluid following the first centrifugation of the crushed suspension) and could not be detected in hydrolysates of the uninoculated medium used for

cultivation of the bacteria. Free heptose was not found in the supernatant after trypsin digestion but was readily demonstrated in hydrolysed samples of it together with amino acids and traces of glucose.

In another experiment whole cells from strain L44 were treated with trypsin. The treated cells were Gram negative. Some cells were slightly swollen but with clear margins and cell fragments or other evidence of disintegration were not found. The trypsin digested cells were then hydrolysed by boiling in 3 N hydrochloric acid for 2 hrs. and the evaporated hydrolysate examined for neutral sugars by paper chromatography. Only traces of heptose were demonstrated while a strong heptose spot was obtained with a hydrolysate of whole cells from strain L44 treated with tris buffer.

The experiments performed show that the aldohexose is part of an outer cell wall layer.

Quantitative Analyses

The results of the quantitative analyses have been compiled in Table 3. The findings varied somewhat from one batch to another, in particular the values obtained for amino acids and lipid in the undigested walls. In a few preliminary experiments the lipid content of the undigested walls from strains L44 and L49 was estimated by ether extraction of hydrolysed wall samples (13). The lipid content estimated by this method varied between 8 and 16 per cent. In order to give reproducible results this method required large amounts of walls (more than 20 mg each sample). The micromethod of Snyder & Stephens was therefore used in the further experiments.

D-glycero-D-galacto-heptose was used as standard in the Dische reaction.

As shown in Table 3 the wall preparations from the filamentous organism contained more sugar but less amino acids than the walls from *I. buccalis*.

TABLE 3

Quantitative Analyses on Cell Walls from the Filamentous Organism and *I. buccalis*
(Per Cent of Dry Weight)

	Filamentous organism				<i>I. buccalis</i>	
	L44 u	L44 t	L49 u	L49 t	L44 u	L44 t
Amino acids	52	21	20	2	70	46
Neutral sugars (as glucose)	20	35	23	4	10	21
Rhamnose	5	19	4	1		
Heptose	7		6			
Hexosamines (as glucosamine)	3	5		4	5	7
Lipid (as tripalmitin)	21	4	18	3	13	5

The values listed are mean values from examination of two or three different batches.

No correction has been made for water uptake during hydrolysis of undigested walls. t = trypsin digested.

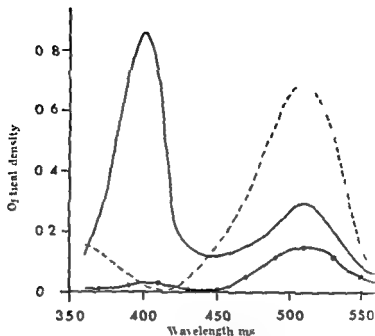


Fig. 1

Dische reaction spectra given by ——— 500 μ g cell walls from the organism, strain L44 ●—● the isolated heptose read against a paper of the same chromatogram - - - - - 100 μ g D glycyrrhizic acid

In order to confirm this chromatographic finding the isolated heptose was subjected to the sulphuric acid oxidation of Dische. The isolation was performed in the following manner: 3 N hydrochloric acid wall hydrolysate (strain L44) made up in distilled water and passed through a column of A IR-120 in the hydrogen form. While the amino acids and imines were retained by the resin the neutral sugars went straight through the column. The fractions containing the sugars were evaporated *vacuo*, re-dissolved in a small volume of distilled water and subjected to paper chromatography in solvent system (C). After having treated different sugars by treating small radiate strips of the dried chromatogram with aniline hydrogen phthalate the heptose was eluted from the paper with distilled water.

The absorption spectra of the chromogens produced in the Dische reaction by the isolated heptose and a sample of undigested walls from strain L44 are shown in Fig. 1. The high optical density at 400 mμ produced by the wall sample is due to rhamnose and glucose which produce absorption maxima at 400 and 410 mμ respectively.

The aldheptose was only found in minimal amounts in hydrolysates of the protoplasm fraction of the bacterial cells (the supernatant fluid following the first centrifugation of the crushed suspension) and could not be detected in hydrolysates of the uninoculated medium used for

DISCUSSION

The difficulties in obtaining a distinct cell wall layer in the density gradient tube and the varying results obtained by the quantitative analyses indicate that the undigested walls were rather heterogeneous and crude preparations. On the other hand they were free of nucleic acid.

Discrepant figures for cell wall lipid were obtained by ether extraction of hydrolysed samples in preliminary experiments and by estimation through the determination of carboxylic acid ester groups. This may in part be due to some hydrolysis of the lipid with loss of certain groups by the first mentioned method. The micromethod of Snyder & Stephens has been used with success in quantitative examinations on endotoxins (7) but as far as I know has never been used for determination of lipids in bacterial cell walls. The possibility that this method includes groupings in the complicated cell wall structure other than the lipid esters cannot be excluded.

With the above reservations in mind it is nevertheless safe to conclude that the walls of the strains examined in addition to the cell wall glycopeptide or mucopeptide contain both polysaccharide, protein and lipid.

The demonstration of an aldoheptose in the walls of the filamentous organism is interesting. Aldoheptoses have up to now only been found as constituents of polysaccharides extracted from Gram negative bacteria. The heptose found in the filamentous organism is without doubt located in the outer part of the cell wall. It could be removed almost quantitatively by treating whole cells with trypsin and this did not cause any gross alterations in cell morphology. Similar treatment of the isolated walls removed in addition large amounts of protein and lipid (cf. Table 3). Large amounts of protein and lipid together with smaller amounts of polysaccharide were also removed by treating the *I. buccalis* cell walls with trypsin. This indicates that the filamentous organism and *I. buccalis* may contain an outer lipo-protein layer connected with some polysaccharide.

The finding of the same amino acids in cell walls of the filamentous organism as in *I. buccalis* strengthens the view put forward in the previous paper that the filamentous organism represents a new *Leptotrichia* species. This view is not challenged by the demonstration of a different sugar pattern in the walls of the two species. Numerous studies on cell walls have shown that the pattern of amino acids tends to characterize taxa higher than species and that the nature of the sugars (and amino sugars) characterizes species.

It was hoped that the chemical characterization of the isolated cell walls also would provide information on the classification of the genus *Leptotrichia*. Most authors are now apt to include this genus in the family *Lactobacillaceae* tribe *Lactobacillae* (5, 12). The isolation of

endotoxic lipopolysaccharides from *L. buccalis* (1, 6) raises doubts about this view. The finding in the present study of lipid and a low amino sugar content in both undigested and trypsin treated walls is in agreement with previous studies on cell walls from Gram negative species. The demonstration of a few major amino acids in the trypsin treated walls however points more to the Gram positive bacteria. Lipid is known to occur in mycobacteria and corynebacteria and has also been found occasionally in cell walls from streptococci (8). The very low amino sugar content may to some extent be due to incomplete hydrolysis in the *Rondle & Morgan* reaction (4). Further studies are needed to clarify the taxonomical position of genus *Leptotrichia*.

SUMMARY

The chemical composition of undigested and trypsin digested cell walls from an anaerobic oral filamentous organism and from *Leptotrichia buccalis* have been compared. Undigested walls from both organisms contained polysaccharide, protein and lipid. The walls from the two organisms contained the same amino acids. Glucose and smaller amounts of galactose were found in *L. buccalis* cell walls and glucose, rhamnose and an aldohexose possibly D-glycero-D-galacto-heptose in the walls of the filamentous organism. Treatment with trypsin removed great amounts of protein and lipid from the walls and in addition all the heptose found in undigested walls of the filamentous organism.

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SEPARATION AND PARTIAL PURIFICATION OF THE SIXTH, SEVENTH AND EIGHTH COMPONENTS OF HUMAN HAEMOLYTIC COMPLEMENT

By
U NILSSON

Received 6 II 67

Recent work has resulted in the resolution of the classical third component of haemolytic complement into six factors (Nelson *et al* 1966 Wellensiek & Klein 1964 Müller Eberhard 1965 Nilsson & Müller Eberhard 1966 Polley & Muller Eberhard 1966 b) In accordance with a recently proposed nomenclature (Complement Workshop La Jolla 1966) they will here be designated C3 C5 C6 C7 C8 and C9 C3 and C5 in this sense are analogous to β_1C and β_1F -globulin respectively (Nilsson & Muller Eberhard 1965)

The main purpose of this paper is to report a procedure enabling separation and recognition of C6 C7 and C8 as individual factors The distribution of C6 C7 and C8 in fractions obtained by a recent procedure for isolating C3 and C5 (Nilsson & Muller Eberhard 1965) will also be described

MATERIAL AND METHODS

Starting material The euglobulin fraction was prepared from fresh human serum after dialysis against a low ionic strength EDTA buffer as described by Polley & Müller Eberhard (1967c) In brief the procedure comprises the following steps a) extensive dialysis of approximately 1000 ml pooled fresh serum from four individuals against 0.005 M EDTA pH 5.4 b) separation of the precipitated euglobulins by centrifugation and decantation of the supernatant c) careful washing of the precipitate in the EDTA solution of pH 5.4 d) dissolving of the precipitate in 20 ml phosphate buffer pH 7.0 e) adding 800 mg of $NaCl$ f) ultracentrifugation for 1 hour at 26000 \times G g) removal of the bulk of the lipids by flotation

Triethylaminoethyl (TEAE) cellulose chromatography was performed as described earlier (Nilsson & Müller Eberhard 1965) with some modifications Approximately 400 ml TEAE-cellulose packed in a 3 \times 70 cm column was equilibrated with

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0.03 M sodium phosphate buffer of pH 8.1. The euglobulin fraction was dialysed against the same buffer before application. The elution procedure comprised a) an initial washing step with 1000 ml of 0.03 M sodium phosphate buffer pH 8.1 b) a gradient of increasing salt concentration and decreasing pH. The gradient was prepared in an open cone system consisting of a 1000 ml round flask as a mixing chamber and a 500 ml Erlenmeyer flask. The flasks were connected by a siphon. Initially the round flask was filled with 0.03 M sodium phosphate buffer pH 8.1 and the Erlenmeyer flask with 0.25 M monobasic sodium phosphate c) washing with 500 ml 0.25 M monobasic sodium phosphate and d) a washing with 800 ml 0.7 M NaCl in 0.25 M monobasic sodium phosphate.

Hydroxyl apatite chromatography was performed according to the procedure of Tiselius *et al* (1956). As described previously (Nilsson & Mller Fberhard 1965) sodium potassium phosphate buffers of pH 7.9 and of stepwise increasing concentration were utilized in the elution procedure. Buffers of suitable concentrations as measured by their specific conductance per cm at 23 °C were obtained by mixing appropriate volumes of 0.07 M sodium phosphate and 0.63 M sodium potassium phosphate of pH 7.9.

C6, C7 and C8 were separated on 200 ml of loosely packed hydroxyl apatite in a 25 × 40 cm column. The adsorbent was equilibrated with phosphate buffer of a conductance of 6 millimhos per cm and a pH of 7.9. Fractions containing C6, C7 and C8 obtained by TEAE-cellulose chromatography were pooled and applied as such onto the hydroxyl apatite column. This pool had a conductance of approximately 11 millimhos per cm and a pH of 7.5 to 8. The elution procedure comprised four subsequent steps of 600 to 800 ml phosphate buffer of a conductance of 6, 11, 12 and 13 millimhos per cm respectively.

For the separation of C3 and C5 400 ml of loosely packed hydroxyl apatite in a 3 × 70 cm column was utilized. The adsorbent was equilibrated with a phosphate buffer of a conductance of 8 millimhos per cm. A pool containing C3 and C5 obtained by TEAE chromatography was applied without preceding concentration or dialysis. Its conductance was in the vicinity of 7 millimhos per cm and its pH around 7.3. Elution was done in four subsequent steps with 600 to 800 ml phosphate buffer of a conductance of 8, 12, 13 and 14.5 millimhos per cm respectively.

The entire separation procedure was performed at +4 °C. As an additional precaution to prevent inactivation of the factors due to bacterial growth all buffers utilized contained 5×10^{-5} M chloramphenicol. Furthermore all fractions were passed through millipore filters ($\text{Q} = 0.45 \mu$) before any of the chromatographic separation procedures concentration by ultrafiltration or storage at +4 °C. Since chloramphenicol of high OD₂₆₀ was present in the buffers protein analysis was performed with the Folin method.

Disc electrophoresis was performed according to Davis' procedure (1964). Equal aliquots of each preparation were separated simultaneously on two polyacrylamide gel columns. One of the gels was stained for protein the other was cut in 0.1 inch segments which were carefully minced in 0.5 ml gelatin veronal buffer (Mayer 1961). The eluates obtained in this way were analysed for haemolysis activity. After correction for the difference in size of the stained and unstained gel haemolysis activity of the segments was correlated with the protein bands of the stained gel.

Preparative electrophoresis was performed on Perkin blocks according to Mller Fberhard (1960).

Sucrose density gradient ultracentrifugation was performed according to Kunkel (1970).

Haemolytic assay techniques Veronal buffered saline containing 0.1 per cent gelatin (Disco) (Wayer 1961) gelatin veronal buffer was utilized as diluent. 1 AC1a4 α 23 cells were prepared as described previously (Nilsson & Mller Fberhard 1967a). This cell antibody complement complex contains an oxidized form of C2 which is approximately 10 times more active than the non oxidized form of the factor (Olley & Mller Fberhard 1966a). Haemolytic assays were performed in an 0.5 ml reaction volume containing 7.5×10^6 1 AC1a4 α 23 cells and EDTA in 0.01 M concentration.

C5 activity was determined in human serum diluted 1:80 previously depleted of C3, C4 and C5 activity by treatment with 1 M KCN (Dalmasso & Mller Fberhard 1966). C6, C7, C8 and C9 were assayed in mixtures of purified components lacking the factor as a control for C6 was also measured in a reaction mixture of C6 deficient

rabbit serum as described previously (Vilsson & Müller Eberhard 1965; Rother *et al* 1966). Highly purified human C9 (Hadding *et al* 1966) for haemolytic assays was kindly supplied by Dr U Hadding. As a qualitative screening procedure aliquots of chromatographic eluates were tested for reconstituting activity of haemolytic test mixtures deficient in single complement components. Haemolytic assay procedures were also utilized for quantitation of C5, C6, C7 and C8 in serum, serum fractions and purified preparations. Purified components in aliquots containing 2 per cent human albumin (Behringwerke, reinst.) frozen in liquid N₂ and stored at -70 °C served as reference material. Each reference preparation was assigned an arbitrary number of units per ml. For each determination the unknown sample and a freshly thawed aliquot of the appropriate reference were diluted in twofold steps. The haemolytic activity of both was determined under identical conditions in the desired test system. The number of arbitrary units per ml of the unknown sample was derived from the relationship between the amounts of the test and the reference samples giving 50 per cent haemolysis.

RESULTS

Partial Purification of C6, C7 and C8

In addition to the bulk of the C3 and C5 (Vilsson & Müller Eberhard 1965) the euglobulin fraction of human serum was found to contain approximately 90 per cent of the C6, 15 per cent of the C7 and 40 per cent of the C8 haemolytic activity present in the original serum pool. The degree of purification obtained by euglobulin precipitation was approximately 20, 4 and 15 fold for C6, C7 and C8 respectively. In spite of the relatively low yield of C7 the euglobulin fraction was considered a suitable source of C6, C7 and C8 as well as of C3 and C5 since all five factors could be isolated from the same starting material. As described previously (Vilsson & Müller Eberhard 1965) TCAE cellulose chromatography was useful as an intermediate isolation step for the two last mentioned components. This procedure was also found to enable the separation of a portion of C6, C7 and C8 from C3 and C5. A separation of euglobulin by TCAE cellulose chromatography is illustrated in Fig. 1. The bulk of the IgG of the euglobulin preparation was eluted in fractions 10–25 in the initial washing step. The protein eluted in fractions 160–200 at a pH of 6.8 to 6.3 and at a conductance of 6 to 7 mS millimhos per cm has previously been found to contain the bulk of C3, C5 and β_{1H} globulin (Vilsson & Müller Eberhard 1965).

Assay of the eluate for haemolytic activities revealed C6 and C8 in fractions 30 to 250. C11 emerged in fraction 142 at pH 7.9 and at a conductance of 5.4 mS millimhos per cm and trailed into the area containing C3 and C5. As demonstrated by Hadding *et al* (1966) C9 was eluted late, largely separated from the other complement components. Material suitable for the further separation of C6, C7 and C8 was obtained from a pool of fractions 60 to 160 (pool II, Fig. 1). This material was free of C3, C5 and C9 activity and was separated from the bulk of unrelated proteins of the euglobulin fraction. A pool of fractions 167 to 197 (pool IV, Fig. 1) was selected for the isolation of C3 and C5 in the way described previously (Vilsson & Müller

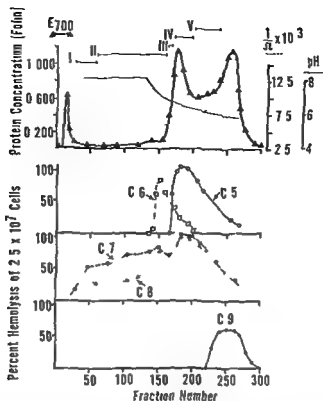


Fig. 1

Distribution of C3, C5, C6, C7, C8 and C9 in euglobulin fractions after chromatography on TFE-cellulose. Gradient started at fraction 50 followed by washing with 0.25 M NaH₂PO₄ at fraction 190 and by 0.7 M NaCl in 0.25 M NaH₂PO₄ at fraction 240. The protein elution pattern is given in the top diagram. The distribution of complement component activities is recorded in the three lower diagrams. Roman figures indicate pools prepared from the eluate as discussed in the text. Pool II was utilized for the further separation of C6, C7 and C8. Pool IV for the further separation of C3 and C5.

Eberhard 1965) The amounts of C6, C7 and C8 of pools II and IV in relation to the total yield of these activities in the chromatographic eluate (pool I—V, Fig. 1) were estimated. Pool II contained 40 per cent of the C6, C7 and C8 activities respectively. Pool IV contained 50 per cent of the C6 activity and 30 per cent of the C7 and C8 activities.

Hydroxyl apatite chromatography was utilized for the further separation of C6, C7 and C8 (Fig. 2). The column was equilibrated with a phosphate buffer of pH 7.9 and a conductance of 8 millimhos per cm. Pool II (Fig. 1) was concentrated by pressure dialysis against the same buffer and subsequently applied to the column. Elution was performed in four subsequent steps with phosphate buffers of pH 7.9 and of stepwise increasing salt concentration. Following application of the material the adsorbent was washed with a buffer of a conductance of 8 millimhos per cm. Some protein unrelated to the complement

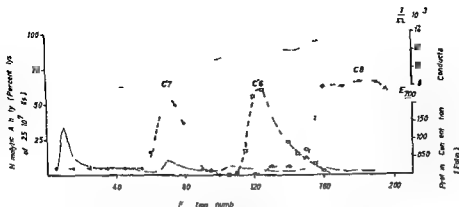


Fig 2

Chromatographic separation of C6 C7 and C8 on hydroxyl apatite. The protein (Pool II Fig 1) was applied in phosphate buffer pH 7.9 conductance 8 millimhos/cm. It was eluted stepwise by buffers of increasing phosphate concentration. The eluate was analysed for protein (—) and for C6 (□ — — □) C7 (● — — — ●) and C8 (▲ — — — ▲) activities.

activities was removed in this manner. After the initial washing step C7 activity was eluted at a conductance of 11 millimhos per cm and C6 at a conductance of 12 millimhos per cm in well separated areas. The main part of the C8 activity was eluted at a conductance of 13 millimhos per cm. Traces of C8 also occurred in the later parts of the C6 area.

Suitable fractions from the chromatogram containing one of the components C6, C7 or C8 without any measurable admixture of the other two were useful as sources of C6, C7 and C8 respectively. Lysis of EAC 1a 4 v2a 3 cells required all three of these factors as well as C5 and C9. Omission of any one of these five complement components from reaction mixtures containing EAC 1a 4 v2a 3 cells resulted in non lytic systems.

TABLE 1
Separation and Partial Purification of C6, C7 and C8

Step 1	Preparation of euglobulin	Precipitated from fresh human serum dialysed against 0.008 M EDTA of pH 5.4
Step 2	TFAE-cellulose chromatography	PO ₄ buffer gradient elution (0.03 M pH 8.1 to 0.25 M NaH ₂ PO ₄)
Step 3	Hydroxyl apatite chromatography	PO ₄ buffer pH 7.9 stepwise elution (conductance 6, 11 M and 13 millimhos per cm)

It was noticed that C7 was eluted to some extent by the starting buffer of a conductance of 8 millimhos per cm (Fig 2). Therefore in later experiments application and initial washing of the column were

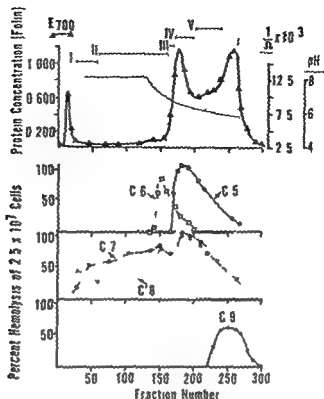


Fig. 1

Distribution of C3, C5, C6, C7, C8 and C9 in euglobulin fractions after chromatography on TP4C-cellulose (gradient started at fraction 50 followed by washing with 0.25 M half phosphate at fraction 190 and by 0.7 M NaCl in 0.15 M half phosphate at fraction 240). The protein elution pattern is given in the top diagram. The distribution of complement component activities is recorded in the three lower diagrams. Roman figures indicate pools prepared from the eluate as discussed in the text. Pool II was utilized for the further separation of C6, C7 and C8 (pool IV) for the further separation of C3 and C5.

(Fberhard 1965). The amounts of C6, C7 and C8 of pools II and IV in relation to the total yield of these activities in the chromatographic eluate (pool I—V, Fig. 1) were estimated. Pool II contained 40 per cent of the C6, C7 and C8 activities respectively. Pool IV contained 50 per cent of the C6 activity and 30 per cent of the C7 and C8 activities.

Hydroxyl apatite chromatography was utilized for the further separation of C6, C7 and C8 (Fig. 2). The column was equilibrated with a phosphate buffer of pH 7.9 and a conductance of 8 millimhos per cm. Pool II (Fig. 1) was concentrated by pressure dialysis against the same buffer and subsequently applied to the column. Elution was performed in four subsequent steps with phosphate buffers of pH 7.9 and of stepwise increasing salt concentration. Following application of the material, the adsorbent was washed with a buffer of a conductance of 8 millimhos per cm. Some protein unrelated to the complement

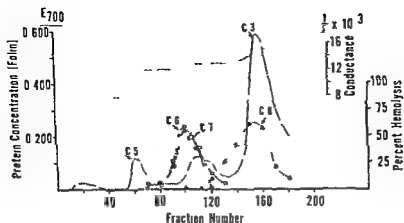


Fig 4

Chromatographic separation of C3, C5 and β_{1H} globulin on hydroxyl apatite. The protein (Pool IV, Fig. 1) which also contained C6, C7 and C8 activity was applied in phosphate buffer pH 7.9 conductance 8 millimhos/cm. The major protein constituents of the material, C5, β_{1H} globulin and C3 were eluted in fractions 56-70, fractions 100-125 and in fractions 150-200 respectively as indicated by the protein curve (—). The distribution of C6 (\square — — \square), C7 (\bullet — — \bullet) and C8 (\blacktriangle — — \blacktriangle) in the eluate was determined by haemolytic assay. Fractions 56 through 70 were utilized for the further separation of C5 by preparative electrophoresis, fractions 150 through 200 for the separation of C3.

purified further by hydroxyl apatite chromatography (Fig. 4). C6 and C7 were eluted together, well separated from C3 and C5. The C8 activity largely overlapped with C3. This contamination prompted an additional purification of C3 by preparative electrophoresis. As illustrated in Fig. 5, C3 and C8 could be separated by this method.

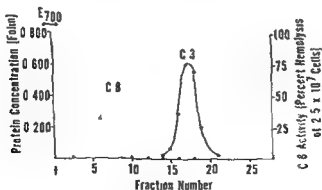


Fig 5

Separation of C3 and C8 (from fractions 150 through 200, Fig. 4) by means of preparative electrophoresis in a Peckham block. A barbital buffer pH 8.6 ionic strength 0.05 was utilized. The voltage gradient was 35 V/cm and the separation was carried out for 71 hours. Application indicated by a slow anode to the right. C8 (\blacktriangle — — \blacktriangle) represented as activity. C3 (\bullet — — \bullet) as protein. No protein corresponding to the distribution of the C8 activity was detected by the Folin method.

C8 migrated anodally as a fast gamma globulin completely separated from C3 in an area where no protein was detected by the Folin method. Partial overlap of C3 and C8 activity occasionally occurred owing to some fast migrating C8 activity but the main part of the C3 area was always free of C8 activity. C8 isolated in this manner was found to become completely inactive within one to three days at +4°C when maintained in the barbital buffer.

Estimation of the Degree of Purity and Physicochemical Characterization of C6, C7 and C8

On a weight basis the purified fractions of C6, C7 and C8 were 1100, 2400 and 200 times more active than whole human serum when assayed specifically for the activity of each of the components respectively. Similar estimates on a preparation of C5 obtained according to the previously described procedure (Nilsson & Muller-Eberhard 1965) indicated a 1400 fold increase of the specific C5 activity.

By physicochemical and immunological criteria the C6, C7 and C8 preparations were heterogeneous. Immunoelectrophoretic analysis of approximately 50 fold concentrates of the three preparations utilizing potent antisera against C3, C5, β_{1H} globulin, IgA globulin and whole human serum revealed no IgG contamination of all three preparations. In addition, C6 and C8 were found to be contaminated with β_{1A} globulin and C7 with β_{1H} globulin.

Disc electrophoresis of equal amounts of the C6 and C7 preparations (approx. 6 μ g protein) in acrylamide gels and 36 μ g of the C8 preparation revealed that all three preparations were heterogeneous. In addition to more slowly migrating protein two distinct protein bands were detected in the C6 preparation (Fig. 3). The faster and larger one migrated at a rate characteristic of the β_{1A} globulin. The smaller one which migrated slower coincided with the C6 active material eluted from the corresponding unstained gel. C7 activity was eluted from an area corresponding mainly to the slower one of two closely situated protein bands. Protein which migrated more slowly than the C7 active material was also visible. Analysis of the C8 preparation by acrylamide gel electrophoresis revealed multiple protein bands. No haemolytic activity was recovered from any region of the corresponding unstained gel.

Analysis of the distribution of the C6, C7 and C8 activities upon sucrose density gradient ultracentrifugation and by Percoll block electrophoresis in barbital buffer, pH 8.6 and ionic strength 0.05, furnished some preliminary information on the physicochemical properties of the components. Analysis of the purified fractions showed a sedimentation rate of 5-6S for C6 and C7 and of 8-9S for C8. Electrophoretically, C6 and C7 were slow β globulins. The C8 activity mi-

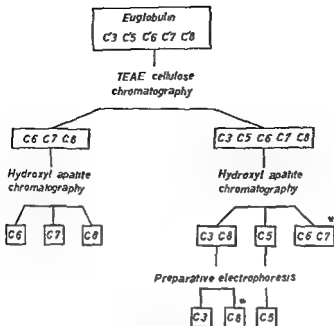


Fig. 6

Outline of the separation procedure for C3, C5, C6, C7 and C8 of the human complement system. Asterisks indicate discarded fractions.

grated as a fast γ globulin but occasionally extended into the slow β region.

Experiments with heat inactivation were performed with purified fractions of C6, C7 and C8. After 30 minutes at 56°C in gelatin veronal buffer C8 was found to be completely and C6 partially inactivated. C7 activity remained unchanged.

DISCUSSION

The procedures for the isolation of C3 and C5 in highly homogeneous form was described in a previous communication (Nal & Muller-Eberhard 1965). In addition a factor of the complement designated C11 was recognized and separated from C3 and C5 in a partially purified form.

The present paper deals with the further purification of the fraction possessing C6 activity and the separation of two additional components of human complement C7 and C8 from this material. The separation procedure which enabled the recognition of these three factors as individual components is based on the previously described method for purifying C3 and C5. The two initial steps of both procedures comprised the preparation from human serum of a euglobulin fraction which was further purified by TEAE-cellulose chro-

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LETTERER SIWE DISEASE

A Survey and a Review of the Course in Two Subchronic Cases

By

KÅRE NYHOLM GEORGE RFTD and KNUD ERIK SJÖLIN

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Letterer Siwe disease has been defined as a distinct pathological entity within the group of proliferative reticuloses. In 1924 *Letterer* (14) described the first case in a paper entitled 'Aleucämische Reticulose – ein Beitrag zu den proliferativen Erkrankungen des Reticuloendothelialen Apparates'. *Siwe* (28) gave an account of the clinical course and the patho-anatomical changes. He characterized the disease as a hyperplastic reticulosis of a non-infectious nature. *Abt & Denenhol* (1) named the described nosologic entity '*Letterer Siwe disease*'.

Our knowledge of the disease originates almost exclusively from a great number of casuistic reports. These reports in addition to demonstrating the continual occurrence of the disease illustrate the numerous difficulties in the differential diagnosis between both benign and malignant diseases of the reticulo-endothelial system. Among the latter there is reason to point out: 1) Infectious reticulosis, 2) aleukemic monocytic leukemia, 3) diffuse reticulosarcomatosis, and 4) eosinophilic xanthomatous granulomatosis of the types '*eosinophilic granuloma of bone*' and '*Hand-Schüller-Christian disease*'.

Clinical and pathological experience gained from work with the latter granulomatosis led to the view of their relationship with the general reticulosis of the Letterer-Siwe type. This found expression in *Lichtenstein's* (16) introduction of the generic term '*histiocytosis*', comprising the '*eosinophilic granuloma of bone*', '*Hand-Schüller-Christian disease*' and '*Letterer-Siwe disease*'. *Lichtenstein* based his conclusion on the transitional forms described.

Though this view obtained extensive recognition, a few workers have raised objections to such a simplification. *Siwe* (29) stressed that the pathologist is only able to find different pathological changes within the same reticulo-endothelial system; he is not therefore justified in concluding that these indicate different stages of the same disease and consequently a common pathogenesis. *Olani* (23) supported *Siwe's* view.

and emphasized the great difference between the clinical manifestations of the disease. The eosinophilic granuloma presents itself as a localized granulomatosis running a predominantly benign course whereas Letterer-Siwe disease is a diffuse reticulosis of toxic character generally running an acute or subacute lethal course.

The object of the present paper is a detailed account of the symptomatology, course and morbid findings in two typical cases of Letterer-Siwe disease, this pathological entity having been described only once by a Danish writer within the past ten years. In addition we have desired to point out the continued existence of the classical general and chiefly non-lipid pathological picture.

REPORT OF CASES

Patient 1 (S14 Dept. of Path., Stanford University School of Medicine No. 3F 199)

A 4 month old boy. There was no familial predisposition. His father was a farmer. His mother a 19 year old primipara (housewife) had been extremely nauseated and vomited several times within the first trimester of pregnancy. She gained about 70 kg during pregnancy. The infant was born at term in a hospital. Delivery was uncomplicated. Birth weight 3.9 kg. The infant and mother were discharged three days after delivery.

The infant was born with a dry, scaly exanthem of the scalp and face. At the age of two weeks he began to vomit, this continued despite repeated changes in the diet. The exanthem began to spread at about the age of 2 months to cover the whole body including palms and soles of the feet two months later. The skin became increasingly brittle and bled easily. During the first 3½ months the infant gained 1.5 kg, he then lost weight and was referred to a dermatologist. Three skin biopsies led to a diagnosis of histiocytosis. At 4 months of age the infant was admitted to the hospital with a diagnosis of histiocytosis.

General state on first admission—The patient was small, well developed and extremely irritable. Weight 5.3 kg, height 66 cm, B.P. 70/50, pulse 120, respiration 20. The skin was dry with a diffuse scaly erythematous exanthem, most pronounced on the scalp, face, trunk and arms. Otologic examination revealed a yellow green discharge in the left auditory canal, the left tympanic membrane was not visible. The right auditory canal and tympanic membrane were normal. The alveolar ridges of the oral cavity were grey and brittle. The lungs and heart were normal. The liver was palpable 2 cm below the right costal margin and the tip of the spleen below the left costal margin. There were bilateral hydrocele and a reducible right inguinal hernia. The extremities presented no changes. Neurological examination was unremarkable.

Laboratory Tests—Hemoglobin 10.9 gm per cent, hematocrit 34 per cent, leukocytes 17,000 including 54 per cent leukocytes with segmented nuclei and 10 with rod shaped nuclei. Microscopy of urine showed a few erythrocytes and leukocytes.

X-ray Examination—The lungs demonstrated diffuse infiltrative processes which were interpreted as reticulosis of the lung tissue. X-ray of the skull disclosed destructive processes in the left parietal sphere and in the right orbital roof as well as questionable changes in the left occipital region. The remainder of the skeletal system showed signs of destruction superiorly in the right humerus.

Treatment and Course—Treatment consisted of local skin therapy. The crusts were washed away with Hibsox soap. In addition applications of salicylic acid-sulphur precipitate ointments and Synalar were used. Little effect was obtained. Cytotoxic therapy with Velban was instituted tentatively in intravenous doses of 1-2 mg per kilogram of body weight. The treatment was discontinued after five weeks because the child's condition had deteriorated with aggravation of the skin lesions, progressive enlargement of the liver and spleen, progressive swelling of lymph nodes, aggravation of the gingival affection, loss of weight and increased irritability. His condition was further complicated by acute diarrhea and non-equal

dehydration which was treated with intravenous fluids including human plasma. He developed an urinary tract infection by *Escherichia* and *Proteus* which was suppressed by streptomycin therapy. Permanent inflammation was noticed in both ears with bilateral otorrhea from which *Escherichia* and *Pseudomonas* were cultured.

Laboratory tests during stay in hospital—During the Velban treatment the thrombocyte count remained normal but the hemoglobin level fell to 7.4 gm per cent. In the course of the recurrent infections the leukocyte count rose to a maximum of 23,000 and never fell below 4,000. Blood urea nitrogen remained between 11 and 15 mg per cent. Alkaline phosphatase and SGOT rose transiently but fell again.

After 53 days in the hospital the infant was discharged for continued treatment at home with Synalar ointment, mandelaminic methionine and vitamins as well as pillosolax baths. At the age of 6 months he was started on prednisone treatment in doses of 40 mg daily. One month later the skin lesions of the face and trunk were subjected to X irradiation; the total dose was 600 r. The maculopapular exanthem of the entire trunk, the scalp and the eyelids cleared somewhat and lymph node swelling subsided. About one month later exanthem recurred on the abdominal skin followed within 48 hours by vomiting and a temperature rise to 40.5°C. At the same time pronounced anemia (4.4 gm per cent) and thrombopenia (5,000) were noted. The liver and spleen increased in size. The child was readmitted at the age of 10 months.

General state on 2nd admission—The infant was anemic and moderately indistinct BP 85/60, pulse 140, respiration 30, temperature 37.3°C. Scattered petechiae were noted over the thorax. A seborrheic exanthem was present behind the ears, on the eyelids and scalp and in the intertriginous areas. The ears could not be adequately examined because the slightest touch caused hemorrhage. The lymph nodes were mildly enlarged. The lungs and heart were normal. The liver was palpable 4 cm below the right costal margin and the spleen was felt 3 cm below the left costal margin; there was no tenderness.

Laboratory tests on 2nd admission—The urine was strawcoloured and cloudy, pH 5.0, no protein, sugar or acetone, microscopy 0-1 leukocytes, 2+ uric acid crystals. Reticulocytes 3.4 per cent, bilirubin 0.8 mg per cent total and 0.55 mg per cent direct.

Terminal course—On the second day after admission treatment with packed red blood cells was begun in doses of 10 ml/kg of body weight. A total of 117 ml were transfused. One of two blood cultures performed yielded growth of streptococci and the other was negative. Penicillin was given. The fecal guaiac test was negative except for two questionable reactions the day before death. Despite transfusions the hematocrit value fell to 14 per cent on the ninth hospital day. Seventy milliliters of packed red blood cells were transfused at that point; the hematocrit rose briefly to 20 per cent but had already fallen to 20 per cent within 24 hours. From the fifth day after admission increasing difficulty in breathing was noted together with distention of the abdomen and edema of the extremities as well as a larval jaundice. The excessively low hemoglobin level raised the question of intraperitoneal hemorrhage. Transfusion was resumed with fresh blood because of the thrombopenia and the infant was treated with digitalis during 24 hours because of increasing cardiac insufficiency.

All treatment failed. The patient's condition deteriorated rapidly with increasing dyspnea, tachycardia and lethargy. An over-all X-ray view of the abdomen disclosed increasing ascites but no distention of the intestines. Electrolyte analysis the day before death showed hyponatremia. The infant died one week after admission after 7 months of illness.

Autopsy—Aug 13 1963

External Examination The body was that of a 17-month-old child with an anemic color of the skin and diffuse hemorrhagic exanthem most pronounced on the face and trunk. The exanthem consisted of massive petechiae and/or conglomerates of small red slightly elevated ulcerations less than 1 mm in diameter. The texture and elasticity of the skin were normal. The skull was normal in shape and the pupils were round and equal. There was mild jaundice of the sclerae. The oral cavity presented no changes. Both auditory canals displayed a slight affection and purulent discharge. A scaly lesion resembling seborrheic dermatitis was noted on the scalp and groins. The thorax was symmetrical and normal. The abdomen was protuberant and distended. The external genitalia were normal.

Internal Examination The abdominal organs were in normal positions. The peritoneal lining was smooth. The peritoneal cavity contained 200 ml of yellowish fluid. There were no adhesions, intestinal obstruction or dilatation. The liver edge reached 3 cm below the right costal margin. Bilateral inguino-crotal herniae were seen.

Thorax The mediastinum presented no changes. The thymus weighed 6 grams, was of buttery consistency and yellow. Petechiae were seen on the epicardium and on both pleural surfaces, most pronounced on the diaphragmatic portion of the pleura. There were no adhesions and there was no fluid in the pleural cavity.

Cardiovascular System The pericardial cavity contained a small amount of fluid. Petechiae were seen in the pericardium and on the surface of the epicardium. The transverse diameter of the heart was enlarged. The heart and lungs weighed 125 grams. The large vessels arose normally. There was moderate concretation of the aorta. A few scattered ecchymoses were noted in the endocardium. The valves and ostia were normal. The foramen ovale was closed. Coronary arteries presented no changes.

Respiratory System The larynx, trachea and bronchi showed no obstructions. The arterial and venous systems were normal. The lung tissue was light red, firm and contained air.

Gastrointestinal System The esophagus was normal as was the gastric mucosa. The pylorus was patent. The remaining gastrointestinal tract, including efferent biliary passages, presented no abnormalities.

Liver, Gallbladder, Pancreas and Spleen The liver weighed 1200 grams. Its surface was normal. Cut surface showed accentuation of the lobular pattern; the color was light yellow. One of the large intrahepatic bile ducts was dilated and filled with green fluid. The right hepatic duct was firm and somewhat irregular. The gallbladder contained clear, slightly yellowish fluid, somewhat dilated, but the passage to the common bile duct was free. The pancreas was firm and white and the cut surface was normal. The spleen weighed 325 grams. It was firm with a single fibrous adhesion on the surface. The cut surface was dark red and homogeneous. No infarctions or tumor masses were seen. The mesenteric lymph nodes were greatly enlarged and mediastinal and inguinal nodes enlarged to a lesser degree. The bone marrow was red.

Reproductive System The kidneys weighed 75 grams each. Remains of fetal lobulation were seen on the surface. The capsule stripped easily. The tissue was rather pale with striate marking in the medulla. The calices were somewhat dilated. Uterus and ovaries were normal. Both ureters were unobstructed. The prostate, seminal vesicles, testes and epididymis presented no gross changes.

Endocrine System The pituitary body was not examined. The thyroid tissue was normal. No parathyroid was found. The suprarenal glands weighed 3 gm each and were well defined. The cut surface was very pale with a very slight content of lipid.

Musculoskeletal System No gross changes were noted. The brain was not examined.

Microscopic Examination (see also Table 1)

The histological changes were characterized by an extensive and varying infiltration by large histiocytes. The cells were round, oblong or spindle shaped, often vacuolated and about twice the size of normal macrophages. The nuclei of these were often oblong, rather light but with no signs of increased activity. There was abundant cytoplasm. A definitely foamy structure was observed only in the histiocyte cells of the thymus.

In the heart, few subendocardial extravasations of blood were seen. The myocardium showed light diffuse infiltration by large mononuclear and spindle shaped cells.

The vessels were normal.

In the liver, fatty vacuoles were seen chiefly periportal (Fig. 1). The nuclei of the liver cells varied in size and chromatin density. There was mild bile duct proliferation and the bile ducts were filled with bile. The sinusoids were hyperemic focally, primarily in the mid zone. Many branches of the portal vein were filled with histiocytes. There was compression of the portal zones and the connective tissue seemed to be compact but not increased. The largest bile ducts in the right lobe were dilated and filled with bile.

The red pulp of the spleen (Fig. 2) was largely filled with or replaced by histo-

TABLE 1
Histopathological Findings

	Pt 1	Pt 2
n	Histiocytic infiltrates subepithelial in the corium Local invasion of the epidermis A few foam cells Focal haemorrhages	Slight focal histiocytosis in the corium Focal ulcerations in epithelium
bone marrow	Narrow cartilage columns Pronounced histiocytic infiltration of the marrow with a few foam cells and giant cells Very few eosinophilic granulocytes Erythrophagocytosis	Pronounced histiocytosis in the marrow Fibrous tissue and large foam cells in focal process in a rib
liver	Stroma fibrous with a few calcified foci and many dispersed histiocytes No foam cells or eosinophiles No Hassall's bodies	Severe fibrosis with scattered collections of lymphocytes and infiltration with histiocytes Focal calcifications No Hassall's bodies No foam cells or eosinophiles
heart	Moderate diffuse interstitial histiocytic infiltration of the myocardium No foam cells or eosinophiles	Massive infiltrates of histiocytes and foam cells in the loose connective tissue and fat tissue around the pericardium Hydropic degeneration of muscle No eosinophiles
lung	Diffuse histiocytosis in pleura and in adventitia of the vessels Focal histiocytosis around the terminal bronchioles No foam cells or eosinophiles	Diffuse histiocytosis in pleura and interstitial tissue No foam cells or eosinophiles
digestive system	Diffuse histiocytosis in the submucosa of the stomach small intestine appendix and colon Histiocytes in subserosal tissue of colon Nests of histiocytes in Peyer's plaques Foam cells erythrophagocytosis but no eosinophiles Ballooning degeneration in plexus mesentericus	Oesophagus: scattered histiocytosis in mucosa Focal histiocytosis in the appendicular submucosa and subserosa No foam cells or eosinophiles Diffuse histiocytosis of mucosa and submucosa in the small intestine Focal histiocytic infiltration in mucosa diffuse in submucosa and subserosa of colon Degeneration of ganglion cells
liver	Periportal fatty infiltration with compression of the portal zones Fibrosis of periportal areas Bile duct proliferation intracapillary cholestasis Histiocytes in the portal venae No eosinophiles or foam cells	Massive periportal fatty infiltration Histiocytosis in the periportal spaces Many histiocytes in the portal veins No foam cells or eosinophiles
Spleen	Histiocytosis in the red pulp and connective tissue A few foam cells no eosinophiles Erythrophagocytosis	Histiocytosis erythrophagocytosis giant cells a few foam cells Extramedullary haemopoiesis

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Digestive system	Diffuse histiocytosis in the submucosa of the stomach small intestine appendix and colon Histiocytes in subserosal tissue of colon Nests of histiocytes in Peyer's plaques Foam cells erythrophagocytosis but no eosinophiles Ballooning degeneration in plexus myentericus	Oesophagus scattered histiocytosis in mucosa Local histiocytosis in the appendicular submucosa and subserosa No foam cells or eosinophiles Diffuse histiocytosis of mucosa and submucosa in the small intestine Focal histiocyte infiltration in mucosa diffuse in submucosa and subserosa of colon Degeneration of ganglion cells
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Spleen	Histiocytosis in the red pulp and connective tissue A few foam cells no eosinophiles Erythrophagocytosis	Histiocytosis erythrophagocytosis and giant cells a few foam cells Extramedullary haemopoiesis

TABLE 1 (cont)
Histopathological Findings

	Pl 1	Pl 2
Pancreas	Histiocyte infiltration in the interstitial tissue No foam cells or eosinophiles	Many histiocytes in the capsule and interstitial tissue in the tail an accessory spleen with histiocytes No foam cells or eosinophiles
Kidney	Numerous histiocytes in the small veins and lymph vessels A focus of histiocytes in the interstitial tissue No foam cells or eosinophiles	Fresh infarct in the cortex Focal histiocytosis in the peripelvic fat and capsule
Urinary bladder		Moderate focal subepithelial accumulation of histiocytes.
Suprarenal gland	Histiocytosis in the reticular zone and round the veins in the medulla. few foam cells histiocytes in the surrounding fat	Local histiocytosis in the capsule
Parathyroid		Diffuse infiltration of histiocytes in interstitial tissue and capsule No foam cells or eosinophiles
Thyroid		Diffuse infiltration of histiocytes in capsule and connective tissue No foam cells or eosinophiles
Hypophysis		Diffuse histiocytosis with foam cells in posterior lobe Granuloma of foam cells in adenohypophysis No eosinophiles
Lymphnodes	Diffuse histiocytosis in sinus and capsule No foam cells or eosinophiles	Diffuse histiocytosis in sinus No foam cells or eosinophiles
Central nervous system	The fat surrounding cauda equina contains infiltrates of histiocytes with many foam cells No eosinophiles	Histiocytes in the adventitia of the meningeal vessels
Middle ear		Connective tissue markedly infiltrated by histiocytes and many foam cells No eosinophiles

cytes The histiocytes varied in size Some contained rather abundant cytoplasm with clear vacuoles while others contained fragments of nuclei and a small number of phagocytized erythrocytes Very few had foamy cytoplasm The white pulp and the lymphatic follicles were not conspicuous No hemorrhages or necroses were seen The esophagus presented no infiltration by histiocytes Below the level of the esophagogastric junction histiocytes were seen scattered through the submucosal layers of the stomach small intestine gallbladder and appendix Peyer's plaques were

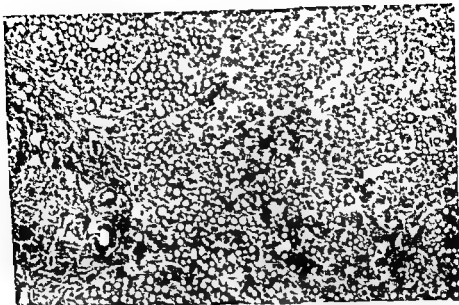


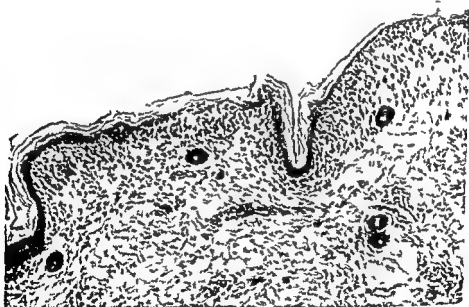
Fig 7 Fatty degeneration of the liver cells especially in the central area

Fig 9 The Spleen Irregular infiltrates with histiocytes in the red pulp is observed

filled with nests of histiocytes and lymphocytes. Peritonitis, along with accumulation of abnormal histiocytes was seen focally.

In most skin areas (Figs 3 and 4) the basal layer of the epidermis was intact. In some places the histiocytes invaded the middle layer of the epidermis but not the granular and horny layers. Focal intradermal hemorrhage were seen immediately below the basal membrane.

Peripheral cells in the glomeruli of the kidney contained hyperchromatic nuclei



Figs 3-4

The skin The subepidermal part of corium is massively infiltrated with histiocytes
The infiltrate is continuous and band like

The tubules were normal with mildly vacuolated cells Numerous histiocytes were seen in the thin walled veins and lymphatic vessels The interstitial tissue and arteries were normal

The pleurae were thickened and contained a large number of histiocytes scattered partly loosely along the interlobar fissures partly in the arterial and venous ad

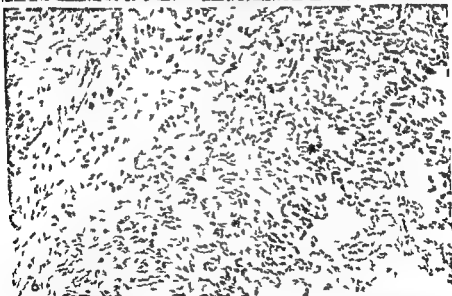


Fig 5 The bone marrow. Most of the myeloid tissue is replaced by histiocytes.

Fig 6 Fibrous adhesion from the right pleura. The tissue is heavily infiltrated with histiocytes and a few lymphocytes and plasma cells. The alveolar septa are similarly thickened.

ventricle. Similar cells were also found in focal arrangement around the terminal bronchioles. In the *suprarenals* histiocytes were present in the reticular zone and around the medullary veins. No foam cells were seen. The *testes* were immature and atrophic. The fibrous stroma of the *pancreas* contained a number of histiocytes. The acini and insular tissue were normal. The fatty and connective tissue of the *thymus*

was diffusely infiltrated by histiocytes. No lymphatic tissue was left. Calcified foci were seen encircled by fibrous rings. The latter were surrounded by mononuclear cells. No normal Hassall's corpuscles were left. The columns of cartilaginous tissue in the bone marrow were abnormally narrow. The spongiosa trabecles in the bone marrow cavity were normal. The marrow (Fig 5) was infiltrated by histiocytes and foci of erythropoid and myeloid tissue as well as megakaryocytes were seen in some places. The spinal cord was normal.

Patient 2 (R.R. Dept of Pathology Stanford University School of Medicine No 3F 19)

A 17 month old girl. No similar diseases were known in the family.

Pregnancy and delivery were uncomplicated. Birth weight 2.6 kg. The infant was sound and developed normally in the first six months of life. She was able to sit at the age of 5 months, to crawl at 7 months, and to walk at 11 months.

Her weight increased normally during the first year of life.

When the infant was 6 months old the mother noted a napkin area dermatitis. At the same time an exanthem occurred on the scalp. This did not respond to ordinary skin therapy. Both lesions were aggravated at the age of 8 months and continued to be resistant to various forms of local therapy.

At the age of 12 months the infant was admitted for a short time to another hospital where a diagnosis of histiocytosis was made on the basis of skin biopsies from the back and genital region. At the same time liver and spleen enlargement were diagnosed. A ray of the thorax revealed a destructive lesion in the left 5th rib. Hemoglobin 10 gm per cent, leukocytes 10,000/cmm.

Steroid therapy was instituted and the affected skin areas were submitted to superficial X irradiation. During the next two months healing was given in doses of 15 mg daily. This was then replaced by Aristocort. The treatment was continued until her admission to the Stanford University Medical Center three months later. During the same period different skin areas were irradiated by a total of 1690 r. At the age of 14 months her development had regressed: she ceased crawling and doing other things learned previously. Her appetite was declining and there was intermittent rise of temperature. Ulceration of the extremities, chronic otorrhea and polypnea were also noted.

She was admitted to Stanford Medical Center at 17 months of age.

General state on 2nd admission.—The infant was malnourished. Pulse 130 B.P. 90/64. Respiration 64. The scalp exhibited scalp exanthem with bleeding areas. The skin of the back displayed a dry scalp exanthem and of the buttocks an erythematous moist exanthem. Bilateral lymph node swelling was observed in the neck. Individual nodes were enlarged up to 3 cm in diameter. There was no axillary node swelling. A greenish discharge was noted in both auditory canals. The patient's face was Cushingoid. The eyes were normal. The teeth were in extremely bad condition. The thorax was normal. The liver was palpable 7 cm below the right costal margin. The spleen was firm extending to 4 cm below the left costal margin. The back and extremities presented no changes.

Laboratory tests. Urinalysis: pH 6.0, specific gravity 1.018, microcopy 1⁺ erythrocytes and 4-6 leukocytes per visual field. No sugar or protein. Hemoglobin 6.7 gm per cent, leukocytes 4000-6000/ μ . Differential: Rod shaped leukocytes 2, segmented leukocytes 40, lymphocytes 49, monocytes 8. Serum electrolytes normal. Bilirubin 1.6 mg per cent direct, 1.2 mg per cent total. Prothrombin time over 100 per cent. Bleeding time 65 min. Serum protein 5.6 gm per cent. Albumin 9 gm per cent, globulin 3.6 gm per cent. Bone marrow aspirate: Histiocytosis and mild erythroid hyperplasia. A ray of the bony system showed a destructive process in the left fifth rib. No other changes were noted in the skeletal system.

Course during stay in hospital.—Of several blood cultures none showed growth of bacteria. A urine sample drawn by suprapubic puncture was sterile. Coagulate negative staphylococci were cultured from the skin and ears. Excessive growth of *Pseudomonas* and *Proteus* as demonstrated in the auditory canals. The treatment consisted of antibiotics for the skin and ear infections as well as local skin therapy in the form of Synalar shampoo and ointment and Cetar shampoo. Buron's solution was used for drying of the napkin area dermatitis. Penicillin and Streptomycin did not alter the course.

After one week in the hospital the hemoglobin level fell to 4.9 gm per cent. Trans

fusion of 100 ml of packed red blood cells treatment for five days with prednisone in doses of 100 mg daily continued X irradiation of the affected skin areas and treatment with Cytotoxan and Velban had no influence on the progressive course. The condition deteriorated two weeks after admission associated with tachycardia tachypnea and increasing ascites. Death occurred 24 hours later during blood transfusion and after 13 months of illness. Artificial respiration and cardiac massage were of no avail.

AUTOPSY

External Examination

The body was that of a 17 month old girl. Weight 7.5 kg, length 70 cm. The scalp exhibited ulcerations and cicatrization particularly pronounced along the hair line. Macerated ulcerations were seen on the skin covering the trunk. The ulcers measured up to 0.5 cm in diameter. Ulcerated areas were red and alternated with scattered petechiae which were particularly conspicuous over the upper part of the thorax and lower part of the abdomen. The vulva was edematous. The skull was normocephalic. The pupils were round and equal. The sclerae were not jaundiced. A greenish mucous discharge was found in both auditory canals and in the oral cavity. The postauricular and submandibular regions were moderately edematous. The thorax and the abdomen were of normal shape. The liver was palpable 3-4 fingerbreadths below the costal margin. The spleen was palpable. The extremities presented no gross changes.

Internal Examination

The Thorax: the mediastinum was fatty. The thymus weighed 6-8 grams. The tissue was firm and fibrous with streaks of yellow buttery tissue. The mediastinal lymph nodes were not enlarged. The pleurae were smooth. The pleural cavity contained a very small amount of serous fluid. A firm fibrous adhesion measuring 1x2 cm extended from the lower part of the left upper lobe to the costovertebral angle.

Cardiovascular system: The pericardium contained 18 ml of yellow visous fluid. The 56 gram heart was not enlarged. The large vessels arose normally. The valves were normal. The foramen ovale was closed. The wall was of normal thickness. The endocardium presented no changes. There was no fibrosis or fresh hemorrhage in the wall. The coronary vessels were normal. Small yellow white intimal plaques were seen in the aorta. The arch was normal. At the descending aorta, a fibrous band 10x2 mm was seen at the site of the closed ductus Botalli. None of the large intrathoracic or abdominal arteries or veins exhibited any changes.

Respiratory system: The larynx, trachea and bronchial tree were grossly normal except for hyperemia of the mucous membrane. An abundant mucous discharge was found in the fairly small bronchial branches. There was no definite obstruction. The arterial and venous systems presented no changes. Lung tissue was only moderately crepitant. Its color was light red apically and darker red basally. On cut section a copious amount of foamy fluid was expressible. Around the left pleural adhesion no changes were found within the lung tissue. The hilar nodes were not enlarged.

Gastrointestinal system: The abdominal organs were situated normally. The peritoneum contained 100 ml of slightly cloudy fluid. There were no hepatic or intestinal obstructions. The pylorus was patent.

The liver: weighed 77.5 grams. The cut surface presented a lobulated, mottled appearance indicative of portal fatty infiltration. The tissue was pale and brownish. The gallbladder was slightly dilated and contained 10 ml of green bile. The mucosa had yellow streaks. The common bile duct was jetted. The intrahepatic bile duct system was normal. The pancreas was normal. The abdominal lymph nodes were not enlarged. The cut surface was normal. The bone marrow was normal. Small foci of white marrow were seen especially in the lower part of the vertebral column. These were presumably infiltrates of abnormal cells. In the left 8th rib a fluctuating semi fluid yellowish mass was found to which the lung adhered. In this area there was osseous destruction and extension of the destructive process into the vertebral body and further around the spinous canal.

Urogenital system The kidneys weighed 32 grams each. Residual focal lobulation was seen on the surface. The color was pale red and cut surface showed the medulla to be somewhat lighter than the cortex. The calyces and pelvis were not dilated. The ureters were free and the bladder slightly dilated. The vesical mucosa presented focal hemorrhages. No changes were seen in the internal genitalia.

Endocrine system The pituitary body was firm. It was 1 cm in diameter. The thyroid, parathyroid and suprarenal glands presented no changes. The cut surface of the suprarenal glands showed the cortex to be pale yellow.

Musculoskeletal system The muscular tissue was firm and red. It was mildly edematous in the upper part of the body. Osteolytic changes were demonstrated in the anterior middle and posterior cranial fossae and in the calvaria these being filled with a soft yellow material.

Central nervous system The brain weighed 850 grams. The gyri were not flattened. The meninges were clear and the vessels normal. The left eye was removed and submitted to microscope which revealed no abnormality. Part of the spinal cord was examined and found to present no morbid changes. The meninges covering the petrous portion of the temporal bone contained nodules of a firm buttery tissue measuring 0.75 cm in thickness. Numerous coronal sections through the cerebrum showed the cortex, thalamus, mesencephalon, pons, medulla oblongata and cervical portion of the spinal cord to be normal with no abscesses, ecchymoses or other abnormalities. There were no intraventricular ecchymoses or petechiae.

Microscopic Examination

Heart The endocardium and myocardium were normal. A coronary artery in the left ventricle exhibited moderate eccentric focal intimal thickening. A focus of intimal thickening was seen in the aorta. This presented atheromatous vacuolation. Histiocytic infiltrates were found in the loose tissue around the pericardium.

Lungs and pleurae The pleurae were thickened, edematous and contained histiocytes. The alveoli contained lymphocytes, macrophages and some residual cell elements. Scattered accumulations of histiocytes were found in the parenchyma some distance from the pleural surface. An increased number of lymphocytes and a small number of leukocytes were demonstrated in the alveolar septa. The bronchi and vessels were normal (Fig. 6).

Massive periportal fatty infiltration was found in the liver cells. There was no necrosis of the liver cells. A few nuclei were vacuolated. The portal spaces contained a moderate number of abnormal histiocytes. In the central areas the liver cells were of normal appearance. Dilated sinusoids were found to contain leukocytes, Kupfer cells and abnormal histiocytes. The walls of the central veins were somewhat thickened. A few normoblasts were seen. In the spleen the sinusoids of the pulp were dilated and contained numerous hemosiderin filled macrophages. Active hematopoiesis was demonstrated. In between histiocytic elements were seen. In some areas they contained vacuoles and in others phagocytized erythrocytes. Many of the nuclei were notched. Others were multilobular and had no peculiar mitotic figures. Here and there were nests consisting of 6-8 cells with round, moderately basophilic and vacuolated nuclei. The cytoplasm was pale blue and well defined. The cells were oblong spindle shaped and 2-3 times the mean size of normal macrophages. The capsule was fibrous but not thickened. The arterioles presented no changes.

The gallbladder was normal. In the tail of the pancreas was an accessory spleen containing infiltrates of atypical histiocytes. The acini and islets of Langerhans were normal. The connective tissue around the pancreatic duct and the vessels was increased in breadth and contained a fairly large number of histiocytes.

Gastrointestinal canal The esophageal submucosa showed lymphocytic infiltrates. The mucous membrane was normal. The stomach, small intestine and colon displayed no microscopic changes. The lymph follicles of the appendix were replaced by focal collections of histiocytes.

Urogenital system The glomerular coils of the kidneys were well defined and the tubules well preserved. The interstitial and vascular tissue were normal where histiocytes were observed in the peripelvic fat.

Endocrine system Moderate accumulation of lipid was seen in the cortex of the suprarenal glands. The reticular zone was fairly rich in cells. The medulla presented no changes. Histiocytic infiltrates were observed in the periglandular fatty tissue (Fig. 7).



Fig 7 Capsule tissue of the adrenal Zona glomerulosa is seen to the left. The capsule and the surrounding fat tissue is infiltrated with a multitude of histiocytes and a few lymphocytes

Fig 8 Tumour like tissue from the middle ear. It consists of connective tissue with massive infiltration of histiocytes many lymphocytes and a few plasma cells

The posterior lobe of the pituitary body was partially replaced by histiocytes. The anterior lobe was normal. The thyroid was without changes. The parathyroid contained histiocytes in the interstitial tissue. The ovary contained numerous oocytes. No Hassall's corpuscles were detectable in the thymus but scattered lymphocytes

infiltrations were seen with moderate fibrosis and diffuse infiltration by histiocytes, as well as focal areas with calcification possibly remnants of Hassall's corpuscles.

In the skin the epidermis exhibited focal ulceration with basophilic debris and infiltrations of leukocytes and lymphocytes in the necrotic areas. There was scant focal infiltration by histiocytic cells in the dermis.

Central nervous system. An increased amount of fibrous tissue was found in the meninges about the petrous portion containing histiocytic infiltrates. Under polarized light the histiocytes were seen to contain anisotropic substance which gave a positive reaction to fat staining. The spinal cord (cervical and lumbar portions), the mesencephalon and the medulla oblongata were normal. Purkinje's cells in the cerebellum were intact. In the pons a few of the neurons were glioid but showed no other definite signs of degeneration. The frontal parietal and occipital cortices and the thalamus were normal.

The bone marrow contained a great number of histiocytes with an amorphous eosinophilic cytoplasm. The marrow of the skull was replaced by histiocytic tumor-like tissue from the middle ear consisted of connective tissue massively infiltrated by histiocytes and several lymphocytes and plasma cells (Fig. 8). At the site of the radiographically demonstrated process in the left fifth rib the marrow was infiltrated by fibrous tissue, large plasma cells and large cells with basophilic cytoplasm and small pyknotic nuclei. The hematopoietic tissue was partially replaced. A small number of megakaryocytes was found.

DISCUSSION

The two cases reported illustrate the subchronic form of Letterer-Siwe disease. The earliest publications (1) considered only the acute form running a fatal course within a few weeks or months.

At Hospital for Sick Children, Great Ormond Street (6) 16 cases of reticulo-endotheliosis were diagnosed within a period of 30 years. Among these were only six disseminated forms (the Letterer-Siwe type). Within the same period 18 lymphosarcomas, 17 malignant lymphogranulomatoses and 91 neuroblastomas were treated at that hospital.

In Denmark 19 cases of Letterer-Siwe disease have been diagnosed within the past 20 years, among which were five atypical forms (20). A few reports from American hospitals (11, 19, 21) give a similar incidence pointing out at the same time the diffuse chiefly non-lipid reticulosis as the rarest form of the triad termed "histiocytosis X".

The earliest characterization of the symptomatology was based on knowledge of the acute forms where the disease was generalized at the time of first admission (1). The subchronic form has been described in subsequent reports (3, 22, 26).

The interest displayed in the initial symptomatology centers particularly on factors contributing towards an earlier diagnosis in the hope of thereby obtaining better results of treatment. Schäfer (27) pointed out the initial occurrence of the skin lesions and subsequent publications (19, 21, 22) largely bore out this observation. In the two patients described above dermatosis of a seborrheic character was the initial sign. In the former this sign was present at birth. In both cases the seborrheic lesions were resistant to ordinary skin therapy whereas it abated in response to subsequent X-irradiation. In the former patient the lesion was complicated by ulcerations and hemorrhage. In both patients the diagnosis was confirmed by skin biopsy (Figs. 3 and 4). This

initial monosymptomatic phase of L S disease is often of several months duration (20-24). Reports in the literature show that the disease is often not diagnosed until it has reached its generalized form at which stage all therapy has been futile thus far. Better results of treatment therefore cannot be obtained unless the disease can be diagnosed at an earlier stage. Accordingly diagnostic skin biopsy is advisable in cases where a lesion of the type of seborrheic dermatitis in infants between 0 and 3 years of age proves resistant to treatment.

Histiocytic and reticular cell infiltrates occur superficially in the corium in lymphogranulomatosis mycosis fungoides, juvenile xantho granuloma and H S C disease among others. A differentiation from the two former types of disease is hardly difficult in practice. Letterer-Siwe disease occurring almost exclusively in the 0- to 3 year age group in which age class the 2 firstnamed diseases are extremely rare.

Whereas dermatosis are almost constantly present in Letterer-Siwe disease and generally from the outset they occur according to Paulson *et al* (24) in no more than one third of patients with Hand-Schüller-Christian disease where they are most often secondary to other signs and symptoms (20).

The clinical appearance and histological picture of the skin lesion is about the same in the two diseases though the more chronic forms of Hand-Schüller-Christian disease may present xanthoma cells in the reticular infiltrates in the corium (24).

The progression of the disease is characterized by bilateral otorrhea and cervical and more general lymph node swelling. Processes of bone destruction are revealed by x-ray examination as sharply delineated rarefactions. The appearance is not distinguishable from the osseous processes in eosinophilic granuloma and Hand-Schüller-Christian disease but unlike the bone lesions in these two diseases localization in the bony system in Letterer-Siwe disease is most often an asymptomatic radiographic finding. In the above two diseases the osseous processes will give early clinical signs and symptoms owing to the rapid growth. This causes dilatation of the medullary cavity and penetration through the cortical substance in some cases resulting in spontaneous fracture. A universally demonstrated affection of the bone system is rare in L S disease radiographically. A more frequent finding is an asymptomatic solitary cyst (as in Patient 2) but in many cases radiographic rarefactions are totally absent in the skeletal system (22). Most of the postmortem findings reported have revealed a generalized but not particularly intense reticulosis in the bone marrow as opposed to a more localized and excessively proliferating granuloma formation in eosinophilic granuloma and Hand-Schüller-Christian disease. These findings also lend support in favor of a difference with regard to the osseous manifestations between the general chiefly non-lipid and the focal xanthomatous form of histiocytosis.

Liver and spleen enlargement are cardinal signs which in the more

protracted forms occur at a late stage (26). They indicate generalization of the disease and are consequently an unfavorable prognostic sign.

The most common laboratory tests are of little aid in the diagnosis. The disease is associated with progressive anemia; blood transfusion has had no more than transitory effect. Whether this is caused by a hemolytic factor is so far unknown. Tipton (17) described a hemolytic and pancytopenic phase of the disease but hemolysins have not been demonstrated thus far (11, 20). However erythrophagocytosis is a common phenomenon in the autopsy material. Terminal jaundice could be explained by the pathological processes involving the liver resulting in compression of the portal spaces (Patient 2). An elevated alkaline phosphatase is found in Patient 2, bears out the obstructive character of the icteric state.

The findings of pigment deposits in the proliferated reticular cells in connection with areas of extramedullary hemopoiesis bear great resemblance to the pathologic findings in hemolytic anemia. They may however be explained by the presence of hemorrhages in the tissues together with displacement of the erythropoietic tissue in the bone marrow.

Moderate leukocytosis is often an initial finding (7, 27). The rise of the total leukocyte count has been attributed to complicating infections. In our case 2 the latter were accompanied by peripheral leukocytosis which gradually changed into mild leukopenia. The frequent leukocytosis in the initial phase has given rise to the question whether the infectious component plays a part as a factor releasing and accelerating the abnormal pathological growth. The terminal phase is marked by a normal leukocyte count or mild to moderate leukopenia (1) even in the presence of infection. Depressed leukopoiesis in this phase may account for the absence of leukocytosis.

Careful bacteriological examinations have revealed numerous complicating infections in infants with Letterer-Siwe disease but it has thus far been impossible to establish their definite responsibility for the extensive pathological changes. Bacteriological examination in the present cases revealed the presence of different organisms in the skin (*Staphylococcus*) and ear tissue (*Pseudomonas Proteus*). In both infants postmortem examination showed bacteria in different organs. Further previous reports on fatal septicemia (3, 5, 8, 10, 26) add emphasis to the hazards of this complication especially in cases submitted to steroid therapy. A few descriptions of a non-lethal course after antibiotic treatment (2, 4, 12) must give added interest in the problem of the significance of these bacterial infections possibly as part of a more complex genesis.

Our two patients were both submitted to steroid treatment. We can not say whether this treatment was responsible for the protracted course. Lasting improvement was not obtainable however not even after administration of an excessively large steroid dose (100 mg prednisone daily to patient 2).

Thus a greater interest should be taken in a combined treatment comprising a large steroid dose and a broad antibiotic therapy. A few reports have been published of favorable responses to this (13, 15).

Cytotoxic therapy has been ineffective so far, having even in some instances aggravated the clinical state or caused leukopenia and thrombopenia (13, 26, 30). These results are in accord with the apparent exacerbation following Velban therapy to our patient. A irradiation of the skin lesions, osseous foci and abdomen has given palliative effect but of only short duration (26). In patient 1 this therapy caused remission of both the exanthem and the lymph node swelling.

Thus the treatment of L. S. disease has given only discouraging results. The disease must still be regarded as almost 100 per cent lethal. Nevertheless a few reports of non lethal courses together with more and more frequent observation of a protracted course after intensified therapy must stimulate continued therapeutic investigation.

In the preceding discussion attention has been called particularly to the demand for an adequate therapy with antibiotics in association with steroid therapy. The necessity of such treatment is evident from the results of the bacteriologic examinations in the two patients described above.

The pathological changes are characterized by proliferation of atypical reticular cells in different organs. The proliferating cells displace the normal cells thereby producing the characteristic changes in various organs. In the skin the infiltrates occur subepidermally with local invasion and ulceration of the epidermis (24). In the bone marrow the hematopoietic tissue is displaced being often seen as islands of active erythroid and myeloid tissue surrounded by dense tracts of histiocytes. In the lymph nodes and the spleen the follicular system becomes atrophic while the sinus and the medulla are subject to varying degrees of reticulosis. In the liver hepatic cells undergo fatty degeneration which is periportal in its primary phase (patients 1 and 2). Infiltrates of histiocytes and an increased amount of connective tissue are often seen in portal spaces. Histiocytic cells may be seen in the sinusoids and in the central veins (patient 2). Scattered histiocytes are often observed in the thymus where however fibrosis may predominate associated with degeneration or total atrophy of Hassall's corpuscles. Atypical histiocytic cells may be found in any organs but are here often scattered along the vessels and in the connective tissue.

The pathological histiocytic cell has been described by numerous writers (1, 28). It may vary in shape, size and appearance but there is almost general agreement about the non malignant character of the cell. The cells may contain small vacuoles but proper xanthomatosis with foam cells containing doubly refractive substance (patient 2) is a rare finding and if present a local phenomenon (20). The cells occur either in well defined infiltrates or in the form of a diffuse infiltration. The changes may be accompanied by presence of giant cells of

atypical appearance extramedullary hemopoiesis necrosis and hemorrhages

The degree of reticular cell phagocytosis is high a fact which often characterizes the picture and which caused *Larquet & Claireux* (9) (1952) to employ the term "hemophagocytic reticulosis"

Otani (21) stressed the highly toxic discase picture and suggested a viral etiology as the cause of the general changes. *Swire* (29) pointed out the resemblance of some of the published cases to leukemia and similar diseases. He suggested an autoimmune mechanism as a possible etiological explanation. *Verman & Dargatzis* (18) pronounced that the classical Letterer-Siwe disease resembles much more a generalized reticular cell sarcoma or monocytic leukemia than a granulomatous disease but this opinion has not been supported by subsequent writers.

The views regarding the etiology thus differ considerably. This is due in part to certain variations in degree of the histopathological changes which in some instances tend toward a malignant picture. This variation may suggest a difference in etiology but may just as well be regarded as evidence in favor of a difference in toxicity. Specific toxins have not been demonstrable so far but the variation in the nature of the bacterial infections seems to indicate that these are secondary although we cannot exclude the possibility that they may activate and accelerate a latent disorder in the RFS.

In the two cases presented here the pathological findings corresponded with the classical picture of the universal changes. Further they accentuated the general attack of the disease on the reticulo-endothelial system.

SUMMARY

The course, results of treatment and postmortem findings in two typical cases of *Letterer-Siwe disease* are reported for the purpose of illustrating the subchronic form of the disease.

The difficulties with regard to differential diagnosis are discussed briefly with special reference to the relation of the disease to the chronic granulomatosis of the *Hand-Schüller-Christian* type and the eosinophilic granuloma of bone.

The small influence of therapy on the course of the disease and the risk of fatal septicemia especially during steroid treatment are demonstrated in the two cases presented and are borne out by cases described in the literature. The conclusion is drawn that the best results may be obtained by combining a large steroid dose with broad antibiotic treatment.

The pathological findings are described in detail and the differences from the xanthomatous picture in *Hand-Schüller-Christian* disease with the eosinophilic granuloma of bone are pointed out.

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STRUCTURE OF THE JUXTAGLOMERULAR APPARATUS IN KIDNEYS SITUATED CENTRALLY AND PERIPHERALLY TO AN EXPERIMENTAL COARCTATION OF THE AORTA IN DOGS

By

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Coarctation of the aorta is a congenital narrowing of the aorta localized in typical cases at the ligamentum arteriosum. If the narrowing is pronounced it gives rise to haemodynamic changes. Hypertension and a high pulse pressure central to the narrowing and a faint or impalpable pulse peripheral to it are pathognomonic of the anomaly (Hudson 1965).

As to the pathogenesis of the hypertension three hypotheses prevail (Edwards *et al* 1965 Hudson 1965).

1 The renal hypothesis. The hypertension is supposed to be due to an increased release of vasopressor substances or a reduced release of antipressor substances from the kidneys because of the haemodynamic changes caused by the coarctation.

2 The mechanical hypothesis. The hypertension is supposed to be due to the increased resistance which the heart has to overcome in order to pump the blood through the narrowed aorta and collaterals.

■ The vascular spasm hypothesis. The altered pulse pressure in coarctation of the aorta is supposed to cause an increased peripheral resistance with consequent elevation of the blood pressure.

In an effort to elucidate these problems we studied the histological changes of the kidneys in dogs with experimental coarctation. In particular we were interested in ascertaining whether given haemodynamic alterations caused specific and uniform changes in the juxtaglomerular apparatus of the kidneys. For a detailed description of the method and results the reader is referred to a publication by one of the authors (Svane 1968).

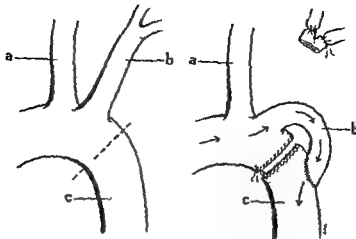


Fig 1

Method for inducing experimental coarctation of the aorta a modification of Blalock & Park's experimental by pass operation a brachiocephalic artery b left subclavian artery c descending aorta

MATERIAL AND METHODS

Young mongrel dogs were subjected to operation establishing coarctation of the aorta autotransplantation of the kidney to the neck and nephrectomy in different sequence and at different intervals. In this way the kidneys were exposed to different but controlled haemodynamic conditions whereupon they were removed and studied histologically.

Measurement of the blood pressure was done by direct puncture of the carotid and femoral arteries under general anaesthesia with 10 mg Nembutal sodium and 12 mg Icopental (pentothal sodium) per kg body weight injected intraperitoneally. The manometer was an inductive transducer with an electronic amplifier. A kymograph was employed as writer. The mean blood pressure is the geometrical mean pressure estimated on the traced curves or measured direct using electrical damping of the pressure curves.

Coarctation of the aorta was induced by a modification of Blalock & Park's (1944) experimental by pass operation for coarctation of the aorta (Fig 1). The left subclavian artery was divided high in the chest; the distal lumen was ligated while the proximal lumen was anastomosed end to side to the aorta which was thereafter divided between the departure of the left subclavian artery and the anastomosis.

Transplantation of the kidney was carried out by a technique whose principle is identical with that described by Pierce & Larco (1964). The kidney was removed through a conventional lumbar incision and placed in a pocket beneath the subcutaneous muscle on the lateral side of the neck and the renal artery and renal vein were anastomosed end to end with the common carotid and the external jugular vein respectively. The ureter was passed through a small incision in the skin more medially on the neck forming a cutaneous ureterostomy.

Histological study The kidneys were cut immediately after removal and a slice of renal tissue a few mm thick was fixed in Helly's solution for 4-6 hours while the remainder of the kidney was fixed in 4 per cent neutral formaldehyde. After fixation the tissue was dehydrated, embedded in paraffin and cut in the usual way. The sections were stained with haematoxylin-eosin, periodic acid-Schiff, haematoxylin (PAS) staining by the method of MacManus or by the methods of van Gieson, Hanzen and Bowie (Bowie 1935; Bang & Kammerer 1961).

The juxtaglomerular apparatus was studied in PAS stained and Bowie stained sections. The juxtaglomerular rating (Schmid & Graham 1967) used has been found to be better suited for the histological assessment of the renin content of the dog kidney (Schmid & Graham 1967; Takats et al 1967) than the calculation of the

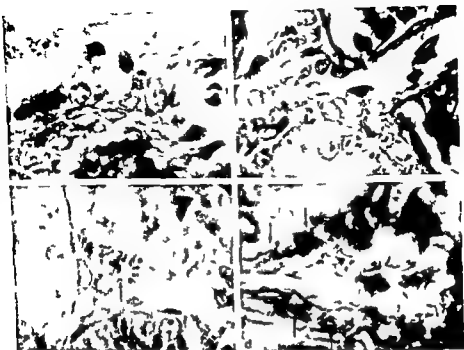


Fig. 2

The juxtaglomerular apparatus in juxtaglomerular rating plus 1 (a) plus 2 (b) plus 3 (c) and plus 4 (d) Bowie $\times 1900$

granulation index used by *Hartoft & Hartoft* (1953) on rat and mouse kidneys. This rating is as follows:

- Plus 1 Normal looking vascular pole with predominantly smooth muscle cells in the arteriolar walls without hyperplasia or increased stainability (Fig. 2a).
- Plus 2 Enlarged vascular pole with increased stainability and with hyperplasia of the cells in the arteriolar media but without cytoplasmic granules (Fig. 2b).
- Plus 3 In addition to hyperplasia of the medial cells distinct cytoplasmic granules in one or two sites in the juxtaglomerular apparatus (Fig. 2c).
- Plus 4 Numerous granules in several sites of the juxtaglomerular apparatus (Fig. 2d).
- Plus 5 Granules showing massive spread or aggregates of—at least one-half—the cells in the juxtaglomerular apparatus.

RESULTS

In accordance with the sequence of the procedures—induction of coarctation, transplantation of the kidney and nephrectomy—the dogs were divided into 4 groups (Fig. 3). The typical alterations in the blood pressure are shown in Figs. 4, 5, 6 and 7 which give the blood pressure findings in dogs T 16, 22, 26 and 30 representing group 1, 2, 3 and 4 respectively. It is outside the scope of the present paper to describe the details of the blood pressure changes, but in outline it may be stated

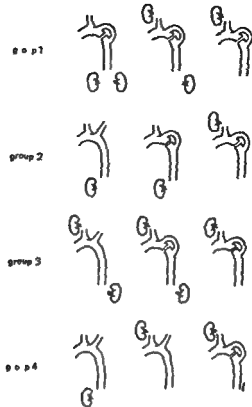


Fig 3

Grouping of the dogs by the sequence of performing the coarctation the renal transplantation and the nephrectomy

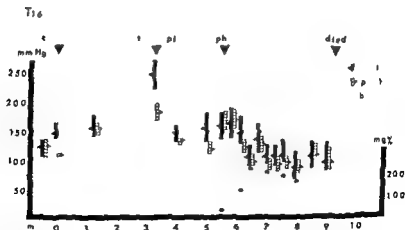


Fig 4

Blood pressure findings in a dog T 16 representing group 1 (Fig 3) The hypertension appearing after the induction of the coarctation disappeared when one kidney was transplanted to the neck and the other kidney removed

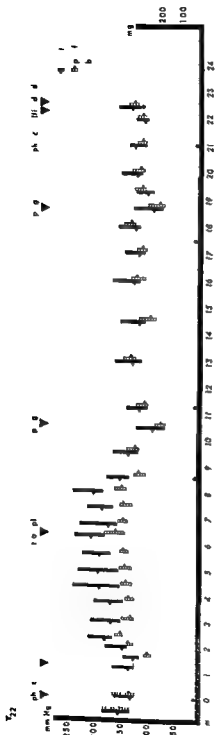


Fig 5

Blood pressure findings in a dog T 22 representing group 2 (Fig 3) After removal of one kidney and induction of coarctation the blood pressure increased but decreased again when the other kidney was transplanted to the neck

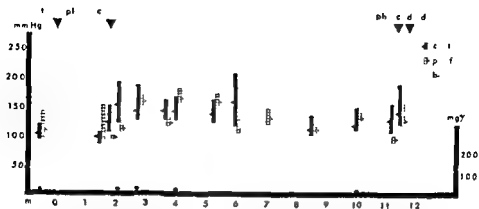


Fig 6

Blood pressure findings in a dog T 9f representing group 3 (Fig 3) After trans plantation of one kidney to the neck and induction of coarctation the blood pressure increased The dog died of uraemia after removal of the abdominal kidney

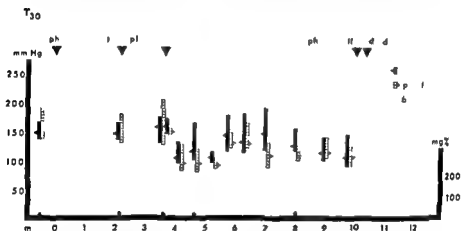


Fig 7

Blood pressure findings in a dog T 30 representing group 4 (Fig 3) After removal of one kidney and transplantation of the other kidney to the neck induction of coarctation did not cause any increase in the blood pressure

1 The blood pressure rises when coarctation is induced and one or both kidneys are placed peripheral to the coarctation

2 This elevation of the blood pressure does not disappear when one kidneys is transplanted to the neck (central to the coarctation) not until the other kidney is removed

3 No elevation of the blood pressure occurs when neither kidney is peripheral to the coarctation at the time that it is induced

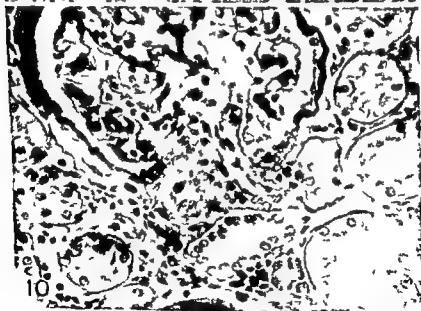
A juxtaglomerular rating was done on 18 kidneys removed from dogs of the 4 groups mentioned at various intervals after the surgical proce

pressures are raised Dog T 25 with a juxtaglomerular rating of 1.2 and 1.1 had—at the first measurement—a moderately elevated mean blood pressure in the carotid artery and a lowered mean blood pressure in the femoral artery Its kidney was removed for investigation 11 days after the induction of coarctation when it died of hemorrhage from the operative site on the aorta Correspondingly the results of repeated blood pressure measurements revealed that in dogs with experimental coarctation and with a kidney peripheral to the coarctation the increase in the mean blood pressure usually appears a few weeks after the coarctation has been induced In the remaining dogs of column b all of which had high juxtaglomerular ratings and elevated mean blood pressures the kidneys had been examined 5–10 months after the induction of coarctation Column c gives the juxtaglomerular ratings in cervical kidneys from 2 dogs with coarctation in which the other kidney had been left in the abdominal site The values are normal i e 1.3–1.5 One of these dogs had a considerably elevated mean blood pressure in the carotid and femoral arteries The other one (T 25—see above) had moderately increased mean blood pressure in the carotid artery but a lowered mean blood pressure in the femoral artery Column d gives the juxtaglomerular ratings (1.5–2.2) in the cervical kidneys of dogs with coarctation whose abdominal kidney had been removed None of the mean blood pressures was elevated

Histological examination of the kidneys showed incidentally in 6 out of 14 abdominal kidneys small interstitial lymphocytic and plasma cell infiltrations quite considerable in one (T 30) In 3 (T 19, 24 and 28) out of 12 cervical kidneys there was severe acute interstitial inflammation with abscess formation (pyelonephritis) All the remaining 9 cervical kidneys exhibited focal parenchymal atrophy and interstitial fibrosis with mild chronic inflammatory reaction One of these 9 kidneys (T 27) showed severe hydronephrosis three (T 16, 25 and 26) mild arteriosclerotic changes with medial hyperplasia in the interlobular arteries and arterioles The remaining 5 cervical kidneys (T 20, 21, 22, 29 and 30) showed no abnormalities apart from the focal parenchymal atrophy, interstitial fibrosis and inflammatory reaction (Fig. 9)

DISCUSSION AND CONCLUSION

In 1939 Goldblatt and his co-workers constricted the thoracic aorta of dogs by a modified renal arterial clamp The experiments failed as the dogs died of hemorrhage but it has been demonstrated by subsequent authors that when the thoracic aorta of dogs is sufficiently constricted the mean blood pressure central to the constriction will increase to hypertensive values in a few weeks Peripheral to the coarctation the mean blood pressure—low immediately after the induction of the coarctation—will increase in the course of the same period to normal or slightly elevated levels Direct measurement of flow and renal



Figs 9-10

- Fig 9 Area from the cervical kidney of dog T 99 showing focal atrophy of the parenchyma, fibrosis and interstitial inflammatory reaction PAS $\times 43$
- Fig 10 Juxtaglomerular apparatus with hyperplasia of cells without granulation in the cervical kidney of a dog (T 30) with a normal mean blood pressure PAS $\times 400$

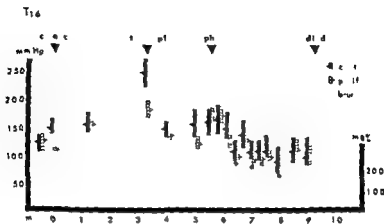


Fig 2

Sequence of the experimental procedures, the time intervals, and the results of determination of blood urea and blood pressure in 5 dogs. (See also Figs 3-6)

sted gross atherosclerotic changes. Table 1 lists the thickness of the aortic wall and the histological findings. In 4 of the operated dogs there was moderate proliferative intimal thickening central to the coarctation never peripheral to it (Fig 7). One control dog had mild intimal proliferation in the ascending as well as the descending aorta. Lipid deposits in the vessel wall were not found in any dog. Fairly mild medial fibrosis was demonstrated central to the coarctation in 4 of the operated dogs peripheral to it in one. One control dog had slight medial fibrosis only in the ascending aorta. The configuration of the elastic lamellae was normal in all the dogs. The total number of elastic lamellae varied somewhat from dog to dog, presumably because they were mongrels, but in all the dogs except in one control it was greater in the ascending

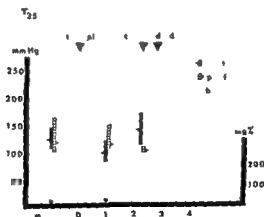


Fig 3
(For text see Fig 2)

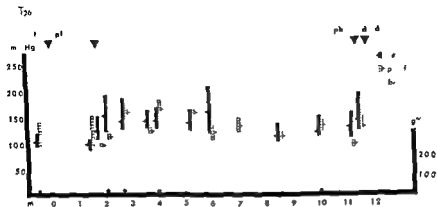


Fig 4
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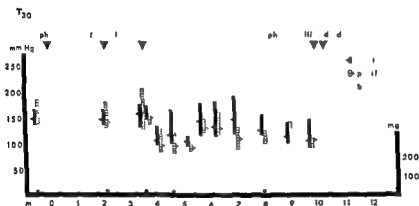


Fig 5
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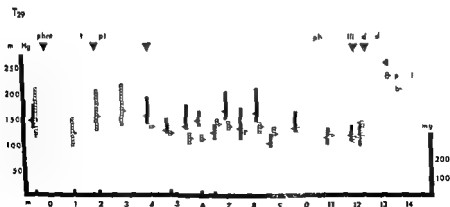


Fig 6
(For text see Fig 2)

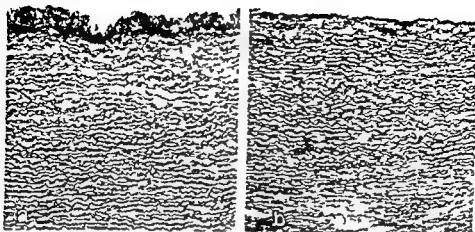


Fig 7

Intimal hyperplasia in the aorta central (a) but not peripheral (b) to the coarctation. Normal configuration of elastic lamellae. T 96 orcein $\times 48$.

TABLE 1

Mural Thickness and Histology of the Ascending and Descending Aorta (Centrally and Peripherally) in 5 Dogs with Coarctation and in 5 Control Dogs

No	Thickness of aorta (mm)		Intimal proliferation (0—++)		Medial fibrosis (0—++)		Number of elastic lam		Number of elastic lam per field		
	asc	desc	asc	desc	asc	desc	asc	desc	asc	desc	
coarctation	T 16	3.0	2.0	0	0	+	+	143	128	17	21
	T 25	3.0	1.3	+	0	0	0	137	100	17	22
	T 26	3.5	1.5	++	0	+	0	159	117	18	22
	T 29	2.7	1.5	++	0	++	0	150	118	16	24
	T 30	2.0	1.5	+	0	++	0	103	90	21	23
control	A	2.5	2.0	0	0	0	0	152	120	20	18
	B	2.0	1.7	0	0	+	0	72	65	18	18
	C	2.0	1.5	+	+	0	0	74	63	21	19
	D	2.0	1.7	0	0	0	0	77	70	20	19
	F	2.0	1.7	0	0	0	0	92	9	18	21

than in the descending aorta. (A direct comparison of the total number of elastic lamellae in the aortae of dogs with coarctation and in controls was not possible because of differences in the size of the dogs.) On the other hand the relative number of elastic lamellae (mean number per field with a dry lens objective $\times 45$) substantially centrally and subadventitiously in the media) was greater in the aortic wall peripheral to the coarctation in all 5 operated dogs (Fig 8) but in only one of the controls. Correspondingly there was a more ample quantity of acid mucopolysaccharides (metachromasia staining with Astra blue) in the

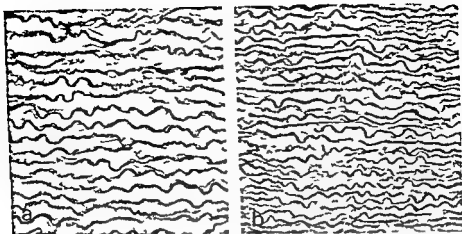


Fig 8

Density of elastic lamellae in the aorta central (a) and peripheral (b) to the coarctation T 29 orcein $\times 190$

aortic wall central than peripheral to the coarctation (Fig 9). Since each aorta was fixed *in toto* this difference must be real—not caused by differences in fixative or fixation period. The number and size of smooth muscle cells in the aorta were estimated to be the same central and peripheral to the coarctation and there was no difference in the amount of PAS positive substance. No calcium deposits were found in the aortic wall.

In other words, there was an increased difference in the mural thickness of the ascending and descending aorta in the dogs with experi-



Fig 9

Metachromatic ground substance in the aorta central (a) and peripheral (b) to the coarctation T 29 toluidin $\times 190$

mental coarctation. This increase was due predominantly to an accumulation of acid mucopolysaccharides in the media of the aortic wall central to the coarctation. In addition intimal proliferation and medial fibrosis were more common in the aorta central to the coarctation than in the ascending aorta of control dogs selected at random. The relation between these changes of the aortic wall and the duration of the experimental coarctation cannot be deduced from the present investigation. However even the dog, T 25 that died 2 weeks after the induction of the coarctation showed an accumulation of acid mucopolysaccharides in the media central to the coarctation but no fibrosis.

DISCUSSION

The aortic changes found in the experimental coarctation in dogs corresponds approximately to the changes of human aorta exhibiting congenital coarctation of the adult type (Heath & Edwards 1959). The mural thickness is usually at a maximum above the coarctation and the configuration of the elastic lamellae is normal. In several cases of adult coarctation however there are aneurysms above as well as below the constriction. In Abbott's opinion (1928) these cases as well as cases showing rupture without aneurysm are due to congenital weakness of the aortic wall.

Dunnill's (1959) results however are to some extent at variance with the present ones. In his 9 cases the aortic wall was usually thicker distal to the coarctation whereas the number of elastic lamellae in the media was greater proximally in 8 out of the 9 cases. An increased quantity of acid mucopolysaccharides was found in the thickened media. However it can hardly be expected that experimental coarctation is fully comparable with the spontaneous human condition in which congenital abnormalities of the aortic wall on either side of the stenotic areas may be operative.

Studying the aortic changes in rats with experimental hypertension (unilateral renal ischaemia) Margarey (1957) found pronounced medial changes with cartilaginous metaplasia in 14.5 per cent of the rats whereas Crane (1962) in rats rendered hypertensive by nephrectomy 11 desoxycorticosterone + NaCl was unable to demonstrate an increased uptake of labelled sulphate in the aortic wall and thus presumably also no formation of sulphated mucopolysaccharides. From arterio-sclerosis research however it is known that sulphated mucopolysaccharides may accumulate in the aorta of experimental animals given adrenaline (Berthelsen 1961, Lorenz 1963) and that with advancing age the composition of mucopolysaccharides in the media of the human aorta changes (Dyrbye 1959, Clausen 1964). These medial changes are an important link in the pathogenesis of aortic arteriosclerosis and are assumed by many students of arteriosclerosis to precede the intimal changes (Thoma 1923, Berthelsen 1964, Lopes de Faria 1965).

That our dogs did not exhibit intimal lipidoses and thus not full blown atherosclerosis must be viewed on the background of the fact that in rabbits *Bronte Stewart & Heptinstall* (1954) could not induce typical atheromatous changes unless the rabbits were simultaneously fed ample cholesterol. Hypertension alone could not induce intimal changes in the rabbits.

As emphasized by *May & Kaplan* (1961) coarctation of the aorta is a naturally occurring 'experiment' illustrating the significance of hypertension to atherosclerosis. In analogy the experimental coarctation described in the present paper seems to be a very well suited experimental model for studying the influence of haemodynamics upon the development of arteriosclerosis.

SUMMARY

The histological changes in the aorta central and peripheral to an experimental coarctation were studied in 11 dogs. All showed thickening of the aortic wall central to the coarctation mainly because of an accumulation of acid mucopolysaccharides in the media.

These changes are in fair agreement with the findings in human coarctation of the adult type and are due to altered haemodynamic conditions. The results might indicate that the elevated pulse pressure above the coarctation is a more important factor in this connection than the mean blood pressure.

The significance of experimental coarctation in arteriosclerosis research is emphasized.

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A QUANTITATIVE STUDY OF MAST CELLS IN APPENDICITIS IN MAN

By

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Received 23 XI 66

It was pointed out already by *Ehrlich* (1877) that mast cells were more numerous in the lower part of the gastrointestinal canal. *Samsonow* (1909) assumed that there was a special type of mast cell in the intestinal canal and that these cells were very numerous on the appendix wall. Mast cells of the normal type were encountered by *Weill* (1920) dispersed profusely through the canal. *Michels* (1923), *Jordan* (1926) and *Bolton* (1933) reported a high mast cell count in the tunica mucosa and submucosa. A correlation was established by *Arvy & Quivy* (1955) between the mast cell count and the histamine content determined by *Feldberg & Harris* (1953) in the wall of the canine intestine. *Lindholm* (1959, 1960) performed quantitative determinations in the wall of the gastrointestinal canal of fetus, man, hamster and rat. A significant decrease was seen in the mast cell count in the lower part of the gastrointestinal canal of the human fetus. In man only the oesophagus and rectum had more profuse mast cells than the other parts of the gastrointestinal canal. The appendix contained 1 205 cells per cu mm. In the hamster the highest mast cell count was in the appendix. A tendency towards a decrease in the mast cell count in the lower part of the gastrointestinal canal was established in both hamster and rat. A large number of mast cells were found by *Godlewski* (1960) in the ileocolic junction and appendix of man.

Ehrlich (1877) and *Westphal* (1880) noted that the mast cell count was lowered in acute and increased in chronic inflammations. It was reported by *Vetchnikoff* (1892) that mast cells helped to remove toxins. *Maximow* (1904) confirmed that mast cells undergo regressive changes in inflammation. It was assumed by *Brack* (1925) that the increase in the mast cell count in chronic inflammatory conditions was indicative of increased connective tissue activity. *Nagayo* (1928) established that mast cells played no active role in acute inflammations but increased in number in chronic inflammations. On the other hand *Quensel* (1933) reported that there were no mast cells in the inflamed tissue itself but on the boundary between healthy and inflamed tissue. It was reported by *Maximow* (1904), *Ernst* (1926) and *Sanyal* (1959)

that mast cells react rapidly in inflammations degenerate and disintegrate McGovern (1957) established that mast cells may play a role in regulating inflammation and Parish (1964) was of the opinion that pharmacologic substances released by mast cells are mediators of changes occurring in inflammation

MATERIAL AND METHOD

The material consisted of 49 appendices from human subjects 12 of them forming the control material. No signs of acute appendicitis were seen in the control material. The specimens were taken from the midmost part of the appendix. The material displayed clear signs of acute inflammation clinically grossly and microscopically. In the cases in which inflammation was spread throughout the preparation the specimens were taken from its midmost part. When gangrene or perforation was present they were taken from adjoining tissue. Both sexes were represented. The preparations were fixed in 4 per cent aqueous solution of basic lead acetate. They were prepared in the usual way and cut in 10 μ sections. Staining was done in 2 per cent toluidine blue aqueous solution for 10 minutes. The mast cells were counted per sq mm in the tunica submucosa. The mean mast cell count was calculated from 5 counted sq mm. A Leitz binocular microscope with a field of vision of 0.066 sq mm was employed. The cells were counted in every other section and the number per cu mm was determined from Floderus's (1944) formula. The final mast cell values per cu mm were scaled down by 44.7 per cent to eliminate the source of error originating in the fixation of the preparations (Lilholm 1959).

RESULTS

Table 1 shows the results for the control material. The minimum per sq mm was 23 the maximum 112 and the mean 55.2 cells per sq mm or 2184 per cu mm after scaling down.

TABLE 1
The Mast Cell Count in the Tunica Submucosa of Human Appendix

Case No	Age years	Number of cells in 5 \times 1 sq mm						Average per sq mm
1	4	56	65	47	66	69		60.6
2	10	45	61	38	56	61		57
3	19	94	71	81	48	56		70.0
4	39	51	57	45	50	19		51.4
5	41	28	23	48	36	17		31.4
6	44	38	32	32	49	4		41.0
7	48	39	46	33	32	38		37.1
8	49	40	60	62	59	41		52.4
9	49	109	89	112	79	72		92.0
10	51	83	39	33	43	45		44.8
11	53	90	94	76	72	75		81.4
12	61	54	46	42	37	24		40.6
								Mean 55
								Average per cu mm 3947
								Average per cu mm reduced by 44.7% 2184

1 The control preparations came from the Institute of Forensic Medicine University of Helsinki and the appendicitis preparations from the Department of Surgery Maria Hospital Helsinki.

TABLE 2

The Mast Cell Count in the Tunica Submucosa of the Inflamed Human Appendix

Case No	Age years	Number of cells in 5×1 sq mm						Average per sq mm
13	7	112	112	140	138	175		125.5
14	7	19	19	21	21	18		19.6
15	10	55	84	70	57	60		64.2
16	11	47	73	56	44	40		49.6
17	11	69	77	57	69	■		63.8
18	11	39	41	47	35	37		39.8
19	17	37	33	24	30	75		29.8
20	13	78	59	88	64	75		77.8
21	16	36	41	50	46	38		47.7
22	17	25	27	77	16	27		23.4
23	19	67	37	47	40	76		43.7
24	21	77	20	18	21	34		24.0
25	25	13	8	10	11	7		9.8
26	27	78	75	45	37	33		37.7
27	27	51	56	57	59	54		55.4
28	28	68	70	62	67	73		68.0
29	34	43	28	75	39	78		37.7
30	35	23	18	17	21	14		18.6
31	36	33	31	31	36	41		34.4
32	6	79	107	114	75	69		88.8
33	16	43	47	39	45	50		44.8
34	25	30	31	19	25	27		26.4
35	26	50	57	60	60	41		53.6
36	26	37	41	34	28	35		35.0
37	29	24	32	21	39	33		29.8
38	31	39	28	27	36	30		33.0
39	36	47	76	73	60	58		67.8
40	48	83	110	113	64	81		90.2
41	56	18	40	46	32	48		36.8
42	10	80	81	59	78	78		71.2
43	21	77	46	56	54	64		59.4
44	25	39	54	36	60	46		47.0
45	26	37	36	47	35	38		37.4
46	39	41	67	68	60	56		57.8
47	65	59	68	56	52	74		61.8
48	66	66	78	50	74	59		65.4
49	67	49	54	43	41	44		46.7
Mean								48.6
Average per cu mm								3471
Average per cu mm reduced by 44.7%								19.0

Table 2 gives the mast cell counts in the inflamed appendix. The results can be divided into three groups. Cases 13-31 are an example of moderate appendicitis with injection, oedema and incipient gangrene. The minimum per sq mm here was 7 and the maximum 140 cells; mean 44.7 cells per sq mm. Cases 32-41 show signs of appendicitis with actual gangrene and perforation. The minimum cell count per sq mm was 18 and maximum 114 cells; mean 50.1 cells. Cases 42-49 display signs of moderate appendicitis with earlier onset and periappendicular abscesses in the history. The minimum mast cell count per sq mm

was 32 the maximum 80 and the mean 55.8. The mean for all the cases of appendicitis was 48.6 mast cells per sq mm or reduced 19.20 per cu mm. The mean age of the control material was 39.8 years and of the investigation material 26.7 years.

DISCUSSION

All appendicectomised patients displayed clinically typical signs of acute appendicitis. The preparations showed signs of inflammation both macroscopically and microscopically. In an acute inflammation tissue oedema with a subsequent increase in tissue volume is seen. This volume growth was not taken into consideration in the present work but tissue shrinkage in connection with the histologic preparation of the specimen was noted and was assumed to be the same for both normal and inflamed preparations. According to the study the cell count per sq mm for a normal appendix was 55.2 and for an inflamed appendix 48.6. Thus the decrease in appendicitis was insignificant.

Earlier workers such as *Maximow Nagayo Quensel, Ernst, McGovern and Sanyal* established a decrease in the number of mast cells in acute inflammation.

Taking the three appendicitis groups separately it appears that the mast cell count was lowest 44.7 in the most acute form of the disease it was 50.1 in phlegmonous and perforated appendicitis when a somewhat longer history can generally be assumed. The mast cell count was highest i.e. 55.8 at the same level as in the control material in the third group which can be regarded as a chronic phase about to become acute. This would seem to indicate that a decrease in the cell count occurs at the very beginning of an inflammation i.e. degranulation. The tissue oedema which develops is probably due to the histamine released by the mast cells. Statistical analysis of the material showed that there was a significant ($P < 0.001$) decrease in the mast cell count in cases 13-31 compared with the control material. It was not possible to show this in the other groups. A counter reaction with possible new formation of mast cells and accompanying increased cell density may be assumed to take place as the inflammation continues. Or is the recovery of the mast cells to be attributed to possible increasing shrinkage of the oedematous tissue? Morphologically no particular difference was seen between the mast cells counted in the control material and those in the investigation material. However it is probable that mast cells play an important role in the regulation of an inflammation by yielding their products to the tissue (*McGovern Parish*).

A way of establishing the cell density more exactly would be to take tissue preparations of equal size from the selected area and then count all the mast cells in the preparation as a whole.

SUMMARY

Mast cells in normal and inflamed human appendix were counted. The preparations were fixed in basic lead acetate cut in 10μ sections and stained with toluidine blue aqueous solution. Cells were counted per sq mm in the submucosa and the cell count per cu mm was calculated from Floderus' formula.

The control material displayed 2184 mast cells per cu mm. In the inflammation material 1920. A significant decrease in the cell count was established right at the beginning of an acute inflammation. Increasing mast cell density was seen in the course of the inflammation. The situation is discussed in detail.

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DIURNAL VARIATIONS IN SPONTANEOUS BODILY ACTIVITY IN HAIRLESS MICE MEASURED WITH ACTIVOGRAPH

By

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and GEORGE VÆRLS

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It is well known that the incidence of miloses in mouse epidermis displays a circadian rhythm. The diurnal variations in the epidermal mitotic count is possibly connected to the variation in spontaneous bodily activity. It has been suggested that the blood concentration of adrenaline plays an important role (Bullough 1947-48). At our Institutes we are studying the kinetics of cellular proliferation in the epidermis of the hairless mouse in relation to carcinogenesis and we felt a need to know the rhythms of spontaneous bodily activity in our strain of hairless mice kept under the ordinary conditions in our laboratory.

MATERIALS AND METHODS

Many methods of measuring the physical activity of small animals have been described previously (see for instance Mann 1950 and Wulff Rasmussen 1953). However these methods have not provided measurements that are truly representative of the spontaneous activity. They have not registered activity due to eating, grooming or other such limited movements. Some of the so called tambour mounted cages are very sensitive but the results are usually given in graphical recordings which are difficult to analyse quantitatively.

We have built an activity recorder on the tambour mounted cage principle in which all activity is integrated over fixed periods. Fig. 1 shows the cage which rests upon four small balloons that are moderately inflated with air. The apparatus was mounted in such a manner that it was in no way affected by vibrations in the building. A rubber tube from each of the balloons is connected to a common membrane box and movements of this membrane are transformed into an electrical signal.

The apparatus is shown schematically in Fig. 2. The activity detector can be divided into a mechanical and an electrical section. The bodily activity of the mice moves the cage on the air cushions. The vertical components of these movements are proportionally transformed to a voltage by the detector. Only signals within a certain frequency range are accounted for. The upper limit of this frequency band is determined primarily by the mass of the cage and the spring constant of the balloons.

This investigation has been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.



Fig 1

The plastic cage with two hairless mice. The cage is resting on 4 balloons moderately inflated with air. In the background to the left is the membrane box.

$$f_{ppr} = \frac{1}{2\pi} \sqrt{\frac{k}{m}}$$

Where m = mass and k = spring constant

The lower limit is determined by the volume of the membrane box and the restriction of the derivating valve in the pneumatic section

$$f_{lwr} = \frac{1}{\sigma r} \frac{1}{R \sqrt{V}}$$

Where V = volume of the membrane box and R = restriction of the derivating valve

By trial the system was so adjusted that neither the upper nor the lower limits were of practical importance for the readings.

In the RMS detector (RMS means Root Mean Square) the signal is rectified and an output proportional to the effective value of the input is generated. This signal is thus also approximately proportional to the movements of the mice. The Integrator is an operational amplifier coupled as an integrator. The output is the time integral of the input after the last reset signal. This output is the impulse (I) of the

corresponding force (F) viz $I = \int_n^t F dt$

The Encoder transforms the data from the analogue to digital form.

At present we use a potentiometer recorder with a circular disc with 120 contact pattern. The Time consists of a synchronic motor with 12 fixed time intervals gives trigger signals to the tape punch and reset the integrator. The results are punched out on standard 8 hole paper tape. On the tape additional information and comments can be recorded manually. The paper tape can later be used directly as input to a digital computer and many different calculations can easily be performed.

The mice were kept in plastic cages and were fed a diet between 1200 and 1400 hr (MFT). The mice were given a complete diet of pellet form and water ad libitum. There was always some food and water available in the cage. The animal

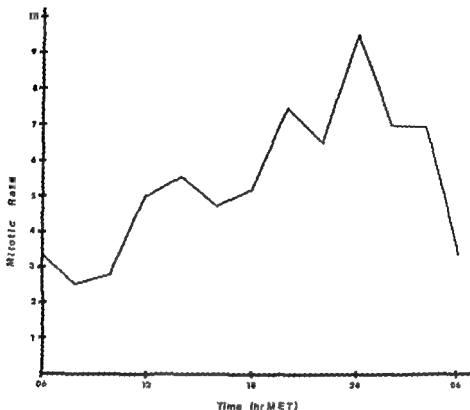


Fig. 4

Circadian rhythm in average mitotic rate by the Colcemid method. The rate is expressed as number of mitoses per 66 mm interfollicular back skin epidermis per hour.

SUMMARY

The paper describes a device for measuring the physical activity of small animals kept under ordinary laboratory conditions.

With this apparatus the diurnal variation in spontaneous bodily activity in hairless mice is registered.

The technical advantages of the system is discussed. The results are discussed with special emphasis on a possible correlation between physical activity and mitotic rate in the epidermis.

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HISTOCHEMICAL STUDIES ON BRAIN PHOSPHATASES IN EXPERIMENTAL LEAD POISONING

By

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Lead poisoning gives rise to dramatic neurological symptoms and yet in routine microscopy these cases usually show only minimal pathological changes in the brain such as patchy nerve cell degeneration and slight vascular endothelial reaction (Pentzschew 1958). These were the alterations revealed in a 2 year old girl (O 39 66 Institute of Pathology Lund) dying from acute lead intoxication owing to a lead button arrested in her stomach.

The effect of lead obviously has to be demonstrated by other means. Since lead belongs to the heavy metals which are known to affect the enzymatic activity and since neurons and vessels showed changes it might be of interest to study some enzymes in these structures.

As no useful material was left from the case mentioned above the problem was studied experimentally. Unspecific alkaline and acid phosphatases in the vessels and neurons respectively were investigated in white rats poisoned with lead.

MATERIAL AND METHODS

Twenty nine white male and female rats (Sprague Dawley strain) 6 weeks old and weighing about 120 g at the beginning of the experiment were taken from six litters. They were fed on a standard diet consisting of commercial pellets and water ad libitum. Eighteen animals received on each injection 10 or 20 mg water free $Pb(NO_3)_2$ per 100 g body weight solved in aqua redest in an amount to adjust the administered volume to 1 ml. In all 100 to 200 mg $Pb(NO_3)_2$ per 100 g body weight was given. The animals were injected subcutaneously on consecutive days except for Sundays a new site of injection being chosen every day to avoid necrosis and scarring possibly interfering with resorption of the solution. The controls the remaining eleven rats were given 1 ml saline subcutaneously.

The animals which received lead had a greater weight increase than the control animals in spite of loss of subcutaneous fat. This may be due to the larger amount of faeces found in the bowel.

The animals were decapitated the day after the last injection except for two killed after 9 and one after 15 days. The brain was removed immediately and sliced 2 mm wide were taken one through the basal ganglia—hippocampus and one through the central portion of cerebellum—pontine nucleus from each animal at identical sites. The slices were immediately frozen in solid carbon dioxide or a mix-

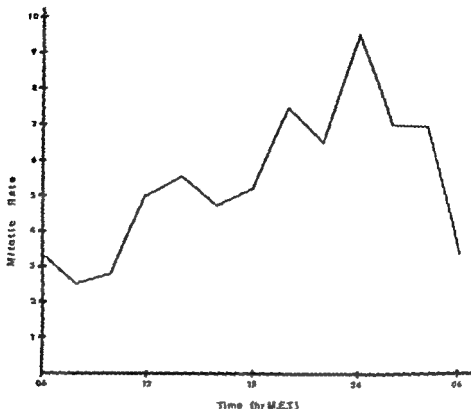


Fig. 4

Circadian rhythm in average mitotic rate by the Colcemid method. The rate is expressed as number of mitoses per 6.6 mm interfollicular back skin of dermis per hour.

SUMMARY

The paper describes a device for measuring the physical activity of small animals kept under ordinary laboratory conditions.

With this apparatus the diurnal variation in spontaneous bodily activity in hairless mice is registered.

The technical advantages of the system is discussed. The results are discussed with special emphasis on a possible correlation between physical activity and mitotic rate in the epidermis.

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ture of propane pre-pylene chilled with liquid nitrogen and cut at -20°C in a cryostat (Dittes Duplicator) in 10μ thick sections transferred to cover glasses.

Sections to be stained for enzymes were fixed for 30 min in acetone at -20°C in the cryostat and thereafter for another 30 min in acetone at room temperature. Sections to be used for routine staining were while still wet fixed at room temperature in a mixture containing ethyl alcohol, acetic acid and formalin (8:5:10).

For the demonstration of acid and alkaline nonspecific mono phosphatases a simultaneous coupling azo technique was employed using Naphthol AS TII phosphatase as a substrate (Burstone 1958).

Acid phosphatases were demonstrated at pH 5.0 with fresh diazotised pararosaniline (Barla & Anderson 1963). A 0.1 M tris maleate buffer was used. The sections were incubated for one hour at room temperature and at 37°C .

Alkaline phosphatases were demonstrated at pH 9.2 with Fast Violet 1B (Sigma) as a diazosalt. The buffer was a 0.1 M tris maleate. The sections were incubated for 20 min at room temperature.

After staining the sections were washed carefully and some counterstained for 45 sec in Mayer's Haematoxylin. All sections were mounted in glasdone C (Burstone 1957).

Alternate sections were used for routine stainings such as Nissl, Mahon for myelins and Haematoxylin and Eosin after ethyl alcohol, acetic acid, formalin fixation. The sections then were dehydrated and mounted.

In 10 cases those parts of the brains not used for cryostat cutting were analysed for lead with a spectrophotometric dithizon method in use at the Clinic of Occupational Medicine, University Hospital of Lund. For valuable assistance with this analysis we owe thanks to Dr B. Fristedt, engineer Övrum and colleagues.

Sections from experimental and control animals were incubated in the same dish. Changes in enzyme activity of the experimental animals were evaluated in reference to the over all activity in the control animal. In addition localization of the activity of acid phosphatases within the cell and in different cell groups was studied and compared with that of control animals.

The alkaline phosphatase present mainly in capillaries and choroid plexus but also found in the central gray matter was evaluated according to the over all activity. The acid phosphatase present mainly in nerve cells of the hippocampus, basal ganglia, pontine nuclei, cerebral cortex and purkinje cells was judged in the same manner with special attention paid to the two last mentioned structures.

RESULTS

Routine histology (Mahon, Nissl, Haematoxylin and Eosin) showed mild to insignificant changes of the type referred to in the introduction, namely discrete focal loss of neurons in the cortex. The surrounding microglia showed a moderate proliferation and the endothelial cells in capillaries and precapillary vessels were slightly swollen. There were no convincing signs of brain oedema.

Quantitative lead analysis of 10 animals revealed a significant increase in lead in the brain as well as in other organs (liver, kidney, blood). No lead was found in the controls.

Animals killed the day after cessation of lead administration showed a slight decrease in alkaline phosphatase activity in the capillaries in all but one animal where the activity tended to be stronger. Simultaneously the activity of the acid phosphatase in the neurons compared to that in controls appeared to be increased in all 10 animals killed the day after cessation of lead administration. In rats killed 9 days or more after the last lead injection the enzymatic activity did not differ from that in controls. Restoration to normal activity tended to be somewhat slower for acid phosphatases than for alkaline. The impression of an

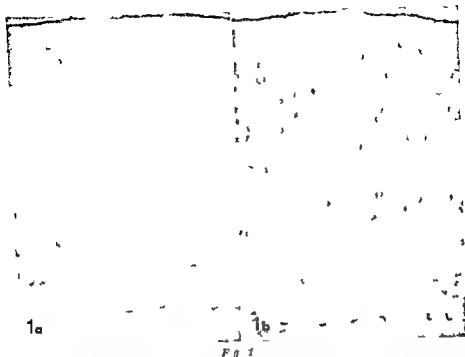


Fig 1

Parasagittal neocortex $\times 63$ a) id phosphatase naphthol AS TR diazotated pararosaniline a control b) lead poisoned rat. Note in b) increased neurophil staining esp in cortical layers II-IV and increased cellular staining esp in layer III and V compared to a)

creased activity of acid phosphatases e.g. in the parasagittal neocortex in lead poisoned animals on closer inspection was found to be due to a stronger staining of the neurophil especially in cortical layers II-III and of the neurons in layers III and IV (Fig 1 a + b). The staining of the neuron cytoplasm was granular in controls and in poisoned animals in addition more diffuse (Fig 2 a + b). Especially the nerve cells of layer V stood out as densely stained (Fig 2 a + b). In the neurons the stained granules tended to be coarser and clumped together in a narrow perinuclear zone of the cytoplasm (Fig 3 a + b). These changes enhanced the laminar distribution of certain neurons (Fig 1 b compared to 1 a). In the purkinje cells these differences were much less marked.

DISCUSSION

Located in the lysosomes the *acid phosphatase* is assumed to play a role in the production of energy and in the protein synthesis and intracellular digestion (Bejll 1966 and De Dure 1966). An increase of the histochemically detectible enzyme activity is not found in axonal reaction but also in electro shock liver coma (Adams 1968) and high age with senile degeneration (Jeschke 1949).

In a study on autolysis in the brain a shift from bound to free activity was noted the total activity remaining constant (Anderson 1960). This shift may be due to rupture of lysosomes and release of enzyme into the cytoplasm. Histologically this may produce a change from normal granular pattern to a more diffuse staining of the cytoplasm. In autolysis Andersen also noted a perinuclear aggregation of granulae.

The apparent increase of acid phosphatase activity and diffuse staining of the cytoplasm found in the present study may be due to an increase of enzyme or a consequence of liberation of the enzyme into the cytoplasm from ruptured lysosomes. More of the cytoplasm becoming stained than normally. We also found the above mentioned perinuclear aggregation of stainable granules.

There are thus similar changes in acid phosphatase distribution in autolysis and lead intoxication. In the latter condition however the changes are reversible and may be interpreted as a sign of degeneration or an increased metabolic effort on the part of the nerve cell analogous to what is seen in the regenerating neuron in the axonal reaction. In this situation however there may be no real difference between regeneration and degeneration the latter being an introductory step and necessary prerequisite for ensuing regeneration. The apparently increased activity may thus be compatible with a reversible degenerative process in the neuron vaguely suggested by routine histology.

The alkaline phosphatase is assumed to partake in the glucose resorption in the brain (Stadtman 1961) and also collagen formation in the body. Its role in the central nervous system however is far from clear.

The reduced activity found in the present study may indicate a toxic inhibition produced by the lead which is known to be toxic to vessels (Pentzsch 1958). With regard to the dependence of the brain on glucose this should be important for the metabolism of the CNS. This hypothetic block may be an important link in a chain of events leading to interference with nerve cell function in lead intoxication. It may also be placed in conjunction with the known microscopic changes revealed by the vessels in lead intoxication, viz. endothelial hyperplasia and arteriosclerotic like changes.

The normalization of the changes in enzyme activity on cessation of lead administration found in the present study indicates that enzyme

Figs 2 & 3

- Fig 2 Parasagittal neocortex Ia rat $\times 160$ a) phosphatase Fig 1 a) control b) lead poisoned rat. Note increased staining of nerve cells in b compared to a.
- Fig 3 Basal pontine nuclear nerve cells $\times 400$ acid phosphatase as in Fig 1 a) control b) lead poisoned rat. Granules concentrated to perinuclear zone of cytoplasm in b.

matic damage caused by the lead was reversible. This is in agreement with the restitution of many clinical symptoms.

CONCLUSIONS AND SUMMARY

Routine light microscopy shows minimal pathological changes in acute experimental lead poisoning, in rat in spite of accumulation of lead in the brain tissue.

Histochemically apparently reversible changes appear consisting of slight reduction in the activity of alkaline phosphatase in the vessels but apparent increase of the activity and changed distribution of acid phosphatases in the neurons. The effect of lead is more easily demonstrated by histochemical methods than by routine microscopy.

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5-HYDROXYTRYPTAMINE IN NORMAL AND SECTIONED RAT SCIATIC NERVE

By

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Received 24 x 68

Mast cells are present both in the epineurial and perineurial coats and in the connective tissue of the endoneurium in peripheral nerves of the rat (Enerback *et al* 1963 Olsson 1965 1966). Rat mast cells have been found to contain several highly active biological substances. The capacity of these cells obtained from other tissues to synthesize and store not only heparin and histamine but also 5-hydroxytryptamine (5-HT serotonin) has been demonstrated by several investigators (for ref see Riley 1959 Smith 1963 Paulsner 1963 Uvnäs 1964 Selye 1965 Bloom 1965). There are strong reasons to believe that the same findings hold true for the mast cells in peripheral nerves of the rat (Olsson 1965).

Recently it has been found that sectioning of the rat sciatic nerve is followed by a very large numerical increase of endoneurial mast cells throughout the distal part of the operated nerves (Enerback *et al* 1964 1965). The aim of the present study was therefore to study the changes in 5-HT in the part of the sciatic nerve distal to a transection using biochemical methods.

MATERIAL AND METHODS

Eleven groups each consisting of four male Sprague Dawley rats were used. Each group comprised rats of the same age weighing between 200 and 475 grams at the end of the experiments. All determinations of 5-HT were done on separately pooled material from the right and left sciatic nerves.

Control rats. Three groups of rats were used as controls. The rats were sacrificed by decapitation without anaesthesia and the sciatic nerve from tube 05 to 13 chi to fossa poplitea were removed immediately.

Experimental rats. In eight groups the right sciatic nerve of the rats was sectioned immediately below tuber osseus 1 chi under anaesthesia. After 3, 4, 6 and 12 weeks respectively the rats were sacrificed by decapitation. The sections of nerves from the scar to fossa poplitea and the corresponding part of the non-operated left nerves were pooled separately and then analysed for their content of 5-HT. In the control and experimental rats the nerves were dissected together with their epineurium and perineurium.

Determination of 5-HT. The 5-HT of the sciatic nerve was determined spectrophotometrically in 3 N HCl after cation exchange chromatography (Berthel 1961 and n & Magnusson 1967).

This work has been supported by the Swedish Medical Research Association against Heart and Chest Diseases and technical assistance by Miss Birgitta Nilsson.

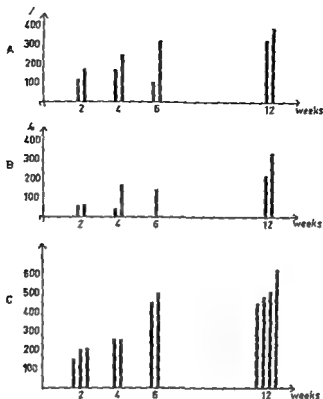


Fig 1

The content of 5 hydroxytryptamine (a) the concentration of 5 hydroxytryptamine (b) and the number of endoneurial mast cells (c) in the distal portion of the right sciatic rat nerve in per cent of that of the same portion of the left nerve. The right nerve was sectioned at the time 0. Each column represents a single determination. The columns at point 0 in A and B represent control determinations in two non-operated rats. X determination not performed.

RESULTS

The distal portion of the left unoperated sciatic nerves contained $0.078 \mu\text{g}$ 5-HT per 4 nerves ($\text{s.e.m.} = 0.0145$ $n = 11$) or $0.53 \mu\text{g/g}$ wet tissue ($\text{s.e.m.} = 0.045$ $n = 11$). Any differences in 5-HT content or in weight between the left sciatic nerves taken from unoperated and operated rats were not observed.

The distal portion of the right sciatic nerves in the unoperated rats contained about the same amount and concentration of 5-HT as that of the left nerve (Fig 1). After sectioning, the 5-HT in the distal segment of the cut nerves displayed a marked and successive increase during the twelve weeks of investigation (Fig 1). Since the weight of the nerve distal to the lesion also showed an increase, the concentration expressed in per cent did not rise to the same degree as the total content.

The increase in number of endoneurial mast cells in the sciatic nerve distal to a lesion is shown in Fig 1c (values from *Enerbacl et al 1969*).

The time course of the increases of endoneurial mast cells and of 5-HT is similar

DISCUSSION

The present study revealed that normal rat sciatic nerves contain 5-HT. The cellular localization of 5-HT in peripheral nerve trunks has previously been studied with the fluorescence microscopic technique of Hillarp & Falck for monoamines (Olsson 1965). In the sciatic nerves of normal rats yellow fluorescence characteristic of 5-HT was observed in the mast cells found in the endoneurium and the surrounding sheaths. Thus it seems most likely that the major part of the 5-HT content in the nerves is derived from mast cells in the endoneurium, the perineurium and epineurium sheaths.

This study also demonstrated that nerve section is followed by a significant increase in the 5-HT content in the distal part 2 to 12 weeks after operation. A large increase in the number of endoneurial mast cells in sectioned nerves during the same period has been observed previously (Enerback *et al.* 1964, 1965). These newly formed mast cells also give histochemical reactions typical for 5-HT (Olsson 1965). These observations strongly suggest that the increased level of 5-HT found in the distal part of sectioned nerves occurs in the numerous endoneurial mast cells present in the degenerating nerves. The finding of a good agreement between the time course of the increases in the 5-HT and the endoneurial mast cells also favours this assumption.

The increase of the 5-HT concentration was however smaller than the increase in the number of endoneurial mast cells. This difference may be due to the fact that the newly formed mast cells are smaller than the normal ones and therefore in all probability contain a smaller amount of 5-HT (Enerback *et al.* 1964, 1965; Olsson 1965). The possibility also exists that the difference is due to different methods of calculation. The biochemical experiments were performed on the whole nerve while the number of mast cells were counted only in the endoneurium. Since the epineurium and perineurium normally contain at least as many mast cells as the endoneurium and as the increase in the number of mast cells after section appears to be restricted to the endoneurium the increase should be smaller in the biochemical experiments.

SUMMARY

The content of 5-hydroxytryptamine in the rat sciatic nerve distal to a transection increased by about 300 per cent during a 12 week period following the lesion. The time course of this increase coincides with that previously obtained for the increase in number of endoneurial mast cells.

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CONGENITAL PRIMARY ADRENAL HYPOPLASIA

By

SVEN LINDGREN

Received 21 VII 66

Adrenal hypoplasia in association with severe malformation of the brain was described already by *Morgagni*. Such adrenal hypoplasia has also been reported in combination with mild development of the pituitary (3 8 14). In this anencephalic form the adrenals are small and their cortex is of adult type. Such hypoplasia has however also been seen in otherwise normal children of mothers with a serum agglutinin incompatible with the infant's red cells (Rh incompatibility) and in children of elderly primiparae or of multiparae above 40 years and of mothers who had toxicosis of pregnancy (6).

During the last two decades some 10 cases of another type of adrenal hypoplasia have been reported. This type is characterised histologically by a foetal cortex of cytomegalic type and differs also clinically from the anencephalic type.

This paper is concerned with a new case of the latter type together with a survey of the literature and certain features suggesting that the condition is hereditary.

REPORT OF CASE

The mother, aged 23 (born 1943) had been admitted to hospital because of tuberculosis in 1951 and had afterwards felt well. Rh negative. No abortion. In 1964 she gave birth to a healthy boy. She was not pregnant again and parturition was expected at the end of January 1966. Routine examination by a private practitioner in December 1965 revealed nothing remarkable. On January 20 1966 he was delivered while on her way to hospital in an ambulance. According to the ambulance officer the child had shown no signs of life and on arrival at hospital 5 to 10 minutes later the child was dead. Tests for toxoplasmosis in the mother (dye test and complement fixation) were negative.

Verf. post (98/66 Malmö). Boy, crown-heel length 39 cm, W 4.1 kg. Skin around nose and mouth cyanotic. The main finding was widespread haemorrhages with bilateral subdural haematomas and purpura of the skin and meninges. Serosal surfaces, surface of thymus and mucosa of the placenta and urinary bladder. The lungs were not aerated. The testes were of normal appearance but were situated in the abdomen. The pituitary was of normal proportions.

It was soon noted that the adrenals were medullary in size. Despite careful removal and thorough examination of all the internal organs no adrenals could be found. The entire material in the region of the kidneys was therefore set aside for examination in serial sections. No other abnormalities were found. The umbilical cord contained three normal vessels. The placenta showed both large and small infarcts.

All of the internal organs except the spleen which weighed 8 g compared with a normal weight of 49 g (1) were small for total body weight.

Author	Age at onset of symptoms	Symptoms	Clin exam	Steroid therapy	Age at death
Sikl 1948	21 days	No appetite Diarrhoea Weight loss Pigmentation Bradycardia	Died on the day of admission to hospital	—	33 days
Geppert et al 1950	Newborn	Vomiting as in pyloric stenosis Pigmentation	17—KS in 24 hr urine 0.12 mg	+	90 months
Deamer et al 1950	Newborn	Vomiting as in pyloric stenosis Weight loss	Hypонатremia Hyperkalemia 0.60 meq	+	10 months
Mac Mahon et al 1957	14 days	Vomiting	Hypонатremia Hyperkalemia 17—KS 0.60 mg	+	3 months
Harlem et al 1957	16 days	Pigmentation	ECG as in hyperkalemia	+	33 days
Mitchell et al 1959 I	21 days	Vomiting as in pyloric stenosis	—	—	7 weeks
Mitchell et al 1959 II	21 days	No appetite Vomiting	Hypонатremia Hyperkalemia 17—KS 0.24 mg	+	Did well
Boyd et al 1960 I	11 days	No appetite Vomiting	—	+	98 days
Boyd et al 1960 II	9 days	Vomiting	Died on the day of admission to hospital	+	12 days
Stempfel et al 1960 II	Case not published primarily				18 hours
Stempfel et al 1960 II	12 days	No appetite Vomiting Weight loss	Hypонатremia Hyperkalemia	+	Did well
Indgren 1966	—	Died from aspiration during delivery			Newborn

owing to the considerable loss of weight of the adrenals in association with postnatal involution.

Though complete absence of adrenals has also been reported especially in anencephalia, careful examination of some large series of

Body weight (g)	Wt of adrenals (g)	Missinger barely an x glom	Irreg foetal x	Cyto megalv	Hypo physis	Other endocr organs	Other malformations
3700	not weighed 10 x 8 mm 12 x 8 mm	+	+	+	normal	normal	—
15800	25 (15 + 1)	+	—	—	normal	no in formation	—
7900	0.50 + 0	+	+	+	no in formation		—
no in forma tion	1	+	+	+	not examined	normal	Slight aortic coarctatic
3700	0.17 + 0.082	+	+	—	normal	normal	macrogenitosom
3100	0.79	+	+	+	normal	normal	—
substitution therapy 1959 1 year of age							
2950	0.69	+	—	+	normal	normal	—
3200	0.9	+	—	+	normal	normal	—
no in forma tion	—				no in formation		
substitution therapy 1960 3½ years of age							
1780	Only micr	+	+	+	Macr normal	See de script on	—

anencephalia has invariably revealed the presence of adrenals (6) Except in a few cases with extensive malformation of the brain or of the lower trunk it must be seriously questioned whether the adrenals ever are really absent

Hypoplasia involves both adrenals in their entirety but particularly one of the two cortical zones which can be distinguished in the foetus. In anencephalia (so called secondary type) the inner or foetal zone is very hypoplastic. In so called primary or idiopathic adrenal hypoplasia the zona glomerulosa is most hypoplastic or missing. But the preserved foetal zone often shows enlarged cells. This type of hypoplasia is therefore known as the cytomegalic type. Our case appeared to be of this type.

A perusal of the literature revealed 11 cases assignable to this category (Table 1). All of the cases had been seen in boys including 3 pairs of brothers (4, 13, 18). This occurrence in brothers suggests the possibility that the condition may be hereditary.

In primary or cytomegalic hypoplasia the weight of the adrenals at autopsy was always small and in all cases except one they weighed less than 1 g. In that case (16) which was seen in a 20 month old boy (case 9) the adrenals weighed 2.5 g. (normal weight about 3 g.). In one case reported by Stempfel no adrenals were found post mortem. This case had not been examined personally by Stempfel and it is not known how thorough the search for the adrenals had been.

In the literature cases the patients lived for days to years. Ours was the only case in which the patient died at birth. Death was probably due to aspiration but the functional disorders due to adrenal hypoplasia might have been a contributory cause.

In the other cases the manifestations of the disease appeared within the first 3 weeks of life in the form of loss of appetite, vomiting, loss of body weight and fairly rapid dehydration. Some cases were clinically misinterpreted as pyloric stenosis and in 2 cases (7 and 13 case 1) explorative surgery was done because of such a provisional diagnosis. In most cases the clinical picture resembled that of Addison's disease with hypotiraemia and hyperkalemia, low concentration of 17 keto steroids in the urine and sometimes pigmentation of the skin. Treatment with NaCl and steroids often produced improvement and control of the symptoms. At 1 to 20 months however some of the patients suddenly went into shock and died. At the time when the case reports appeared in the literature two patients aged 1 and 3½ years were still alive and doing well on substitution therapy.

Of the 11 literature cases the reports included histological descriptions of the adrenals in 8. In most of these there was a broad band of cells resembling those in the inner zone in the normal foetus. In half of the cases the zona glomerulosa was completely missing. The others had only a very narrow outer zone. The inner zone was built up of large cells not arranged in the normal trabecular fashion. The cells were polygonal with abundant eosinophilic cytoplasm and round nuclei. In most cases the adrenals contained some large giant cells with eosinophilic cytoplasm. The diameter of the cells was twice that of normal cells and the nuclei were situated eccentrically. Some cells were very

cular with prominent nucleoles while others were dense and hyperchromatic

The giant cells occurring in 6 of the 9 cases were in good agreement with those described by *Kampster* (11). He found single giant cells in the cortex of all 23 month old foetuses. The older the foetus the fewer the giant cells. Cytomegaly has been found in 6.5 per cent of new borns (5) and in 1.4 per cent of infants up to 4 months of age (1). In the latter series 6 out of 16 had various malformations. Further *Beatty & Hawes* (2) found malformations in 11 out of 11 cases of cytomegaly and *Dhom* (6) in 2 out of 4. Cytomegaly in primary adrenal hypoplasia should probably be regarded as part of the maldevelopment. In the above cases of cytomegaly the adrenals were of normal size in all except 1. Though this child was anencephalic we considered it justified to classify the disease as primary *cytomegalic* adrenal hypoplasia. Cytomegalia which should not be confused with cytomegalic inclusion disease may be a manifestation of incomplete maturity or a return to embryonal cell formation.

This together with the above mentioned occurrence of the condition in three pairs of brothers argues in favour of the theory that the maldevelopment is of hereditary value. If a woman has given birth to a child with adrenal hypoplasia she should be carefully watched during later pregnancies so that hypofunction if any of the adrenal cortex may be discovered and treated in time.

SUMMARY

A baby boy born at term died from asphyxia during delivery. Gross examination post mortem disclosed no adrenals but histological examination of serial sections of retroperitoneal tissue revealed 2 very small adrenals. The permanent cortex was very thin on the left side and practically absent on the right. The foetal cortex was predominant and was built up of irregularly arranged eosinophilic cells and numerous giant cells. The case was diagnosed as congenital primary hypoplasia (cytomegalic type) which should be distinguished from secondary hypoplasia with anencephalia. Eleven cases were traced in the literature among these three pairs of brothers. The condition therefore appears to be a hereditary anomaly which soon produces symptoms of cortical insufficiency. Two of the above 11 patients aged 1 year and 3½ years and receiving substitution therapy are still alive.

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INFLUENCE OF DIFFERENT ANTIGENS ON
CIRCULATING LYMPHOCYTE POPULATIONS

By

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In a previous study a cyclical variation in the composition of the circulating lymphocyte populations in blood and lymph was found after a single subcutaneous injection of pertussis vaccine (Ernstrom & Larsson 1967a). A similar fluctuation in the number of immunocytes in the spleen has been reported after immunization with certain lipopolysaccharides (Britton & Woller 1966). In the present investigation the influence on the lymphocyte populations of two bacterial lipopolysaccharide antigens known to induce production of 19S antibodies was compared with that of two antigens known to initiate a rapid but short lasting production of 19S antibodies subsequently replaced by a synthesis of 7S antibodies (Benedict *et al* 1962 Bauer *et al* 1963). Furthermore the thymic export and import of blood lymphocytes during the primary response to the antigens was investigated by a comparison between lymphocyte content in afferent and efferent thymic blood.

MATERIAL AND METHODS

Totally 30 male guinea pigs were used 11 of which were untreated normal animals. The rest were divided into 4 main groups and were given a single intra-peritoneal injection of one of the following antigens (number of animals and initial body weight in brackets mean \pm SE).

Pertussis vaccine (commercial preparation of the State Bacteriological Laboratory Stockholm Sweden) in a dose of 2 ml per animal corresponding to 60×10^9 bacteria (67 ± 6 g).

Lipopolysaccharide extract from *E coli* (Lipopolysaccharide W *E coli* 055 B5 extracted by Difco Laboratories Detroit U.S.A.) in a dose of 1 mg per animal (18 ± 4 g).

Sheep erythrocytes (fresh erythrocytes washed four times with saline and diluted to a 10 per cent suspension in 0.9 per cent saline) in a dose of 2.5 ml per animal (100 ± 2 g).

Salmonella typhi H (suspension of formalin killed *S typhi* of the strain 1557 rich in the H antigen formalin 0.4 per cent) in a dose of 1 ml per animal corresponding to 14×10^9 bacteria (66 ± 4 g).

In each group 5 to 11 animals were investigated at different intervals after antigen administration. The investigations were performed between 9 a.m. and 4 p.m. without any selection of the different group times. All the animals used were kept under identical care during the experimental period and fed on cabbage turnips carrots and vitamin pellets. Injections of the lipopolysaccharide from *E coli* resulted in death of a few animals 1 day afterwards. The other antigens caused no mortality.

The guinea pigs were anaesthetized with sodium Nembutal (25-50 mg in a 25 per cent solution 1p per 100 g b.w.) The thymus was exposed and a thymic vein severed. Blood was collected in heparinized pipettes (Heparin® Vitrum Stockholm, Sweden) for counting of white cells in a Bürker chamber and for classification of the lymphocytes by their mitochondrial content in supravital preparations. For the latter purpose slides were prepared with a mixture of Janus green B and neutral red in alcoholic solution (see Culling 1957). A small drop of blood was applied to the slide, a cover glass was put on and sealed with wax. The preparations were immediately examined in a light microscope at 1000 \times magnification. The lymphocytes (100 cells/sample) were registered in six classes: cells with 0-5, 6-10, 11-15, 16-20, 21-30 and >30 mitochondria. The mitochondrial content (MC) is correlated to the size of the lymphocytes—low MC corresponds to small lymphocytes, high MC to large lymphocytes (Wisein 1931, Fichtelius & Larsson 1951, Frimström & Larsson 1963).

The right carotid artery was cut near the origin of the thymic arteries and blood samples were taken for the same analyses as mentioned above. The artery was then ligated.

Finally the thoracic duct was dissected free at its confluence with the left subclavian and internal jugular veins using the technique of Reinhardt & Löffler (1957). The duct was incised and a sample of lymph was collected for classification of the lymphocytes by their mitochondrial content according to the technique used for blood. Afterwards the thymus was taken out and weighed and the animals were killed.

For the white cell count in the Bürker chamber 25 mm³ of blood was diluted with 475 mm³ of Tolisson's solution containing methyl violet for staining of the white cells. Totally 783 squares were counted in each sample, each square corresponding to 0.00625 mm². Differentiation was made between mononuclear and polynuclear cells.

A mitochondrial content of 0-10, 11-20 and >20 mitochondria per lymphocyte is denoted as low, medium and high MC respectively. The results were analyzed statistically by Student's *t*-test. The comparison between the number of lymphocytes of different categories in thymic vein and carotid artery blood was performed by statistical analysis of all the differences in the individual animals. The *p*-values <0.05, <0.01 and <0.001 are denoted as almost significant, significant and highly significant respectively.

RESULTS

The composition of the lymphocyte populations in blood and lymph of the normal guinea pigs is shown in Table 1. The difference in number of small lymphocytes per mm³ of blood from the thymic vein and from the carotid artery in the normal animals was highly significant (statistical analysis of the differences from the individual animals).

TABLE 1

Composition of the Lymphocyte Populations in Thymic Vein Blood, Carotid Artery Blood and Thoracic Duct Lymph of Normal Animals. Mean \pm S.E.

	No. of animals	Cells with low MC		Lymphocytes Cells with medium MC		Cells with high MC	
		%	No./mm ³	%	No./mm ³	%	No./mm ³
Thymic vein blood	14	61.8 \pm 0.6	781 \pm 6 ^a	37.4 \pm 0.4	393 \pm 77	7.8 \pm 0.3	34 \pm 4
Carotid artery blood	14	55.8 \pm 0.4	641 \pm 59	39.9 \pm 0.3	458 \pm 41	4.3 \pm 0.2	48 \pm 4
Thoracic duct lymph	13	28.5 \pm 0.6		67.9 \pm 0.7		8.6 \pm 0.2	

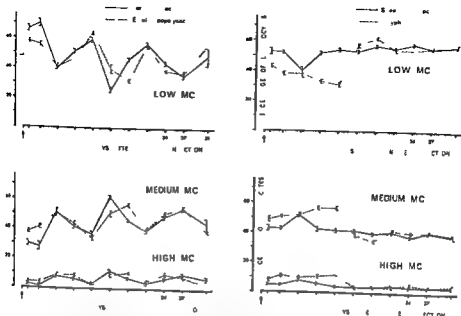


Fig 1

Percentage of lymphocytes with different mitochondrial content (MC) in blood from the carotid artery of guinea pigs given a single injection of pertussis vaccine and lipopolysaccharide antigen from *E coli*, sheep erythrocytes or *S typhi* II antigen. The arrow indicates injection of antigen. Mean \pm SE.

The antigen injections did not influence the normal gain in body weight (about 5 g per day) or the general condition of the experimental animals. An exception was the *E coli* lipopolysaccharide which affected the condition of some animals on the first days after injection and also caused the death of a few animals.

The absolute and relative thymic weights were not significantly changed after the injection of the different antigens.

The granulocytes behaved very similarly after injection of the different antigens. In all four experiments an increased number of granulocytes occurred one day after the injection. After 3 days the number had fallen to about the normal level. The granulocytes then varied in number with maxima at 9-12 and 21-24 days and minima between. No significant difference was present between the number of granulocytes in the carotid artery and thymic vein blood.

Pertussis Vaccine

The percentage and number of small blood lymphocytes (with low MC) increased and decreased cyclically as in a previous experiment when a subcutaneous injection of the vaccine in a lower dose was given (Ernstrom & Larsson 1967). Maxima of percentages were noted 8, 19 and 21 days after injection. The percentage of medium sized and

The guinea pigs were anaesthetized with sodium Nembutal (25–30 mg in a 25 per cent solution 1 p per 100 g bw). The thymus was exposed and a thymic vein severed. Blood was collected in heparinized pipettes (Heparin® Vitrum Stockholm Sweden) for counting of white cells in a Burkner chamber and for classification of the lymphocytes by their mitochondrial content in supravital preparation. For the latter purpose slides were prepared with a mixture of Janus green B and neutral red in alcoholic solution (see Culling 1957). A small drop of blood was applied to the slide a cover glass was put on and sealed with wax. The preparations were immediately examined in a light microscope at 1000 \times magnification. The lymphocytes (100 cells/sample) were registered in six classes: cells with 0–5 6–10 11–15 16–20 21–30 and >30 mitochondria. The mitochondrial content (MC) is correlated to the size of the lymphocytes—low MC corresponds to small lymphocytes high MC to large lymphocytes (Wiseman 1931 Fichtelius & Larsson 1961 Ernstrom & Larsson 1963).

The right carotid artery was cut near the origin of the thymic arteries and blood samples were taken for the same analyses as mentioned above. The artery was then ligated.

Finally the thoracic duct was dissected free at its confluence with the left subclavian and internal jugular veins using the technique of Reinhardt & Yoffey (1957). The duct was incised and a sample of lymph was collected for classification of the lymphocytes by their mitochondrial content according to the technique used for blood. Afterwards the thymus was taken out and weighed and the animals were killed.

For the white cell count in the Burkner chamber 25 mm³ of blood was diluted with 475 mm³ of Toisson's solution containing methyl violet for staining of the white cells. Totally 288 squares were counted in each sample each square corresponding to 0.00625 mm³. Differentiation was made between mononuclear and polynuclear cells.

A mitochondrial content of 0–10 11–20 and >20 mitochondria per lymphocyte is denoted as low medium and high MC respectively. The results were analyzed statistically by Student's *t* test. The comparison between the number of lymphocytes of different categories in thymic vein and carotid artery blood was performed by statistical analysis of all the differences in the individual animals. The *p* values <0.05 <0.01 and <0.001 are denoted as almost significant significant and highly significant respectively.

RESULTS

The composition of the lymphocyte populations in blood and lymph of the normal guinea pigs is shown in Table 1. The difference in number of small lymphocytes per mm³ of blood from the thymic vein and from the carotid artery in the normal animals was highly significant (statistical analysis of the differences from the individual animals).

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	No. of animals	Cells with low MC		Lymphocytes Cells with medium MC		Cells with high MC	
		%	No./mm ³	%	No./mm ³	%	No./mm ³
Thymic vein blood	14	61.8 \pm 0.6	781 \pm 62	37.4 \pm 0.6	393 \pm 33	8 \pm 0.3	34 \pm 4
Carotid artery blood	14	53.8 \pm 0.4	641 \pm 59	39.9 \pm 0.3	458 \pm 41	4.3 \pm 0.2	48 \pm 4
Thoracic duct lymph	13	28.5 \pm 0.6		62.9 \pm 0.7		8.6 \pm 0.2	

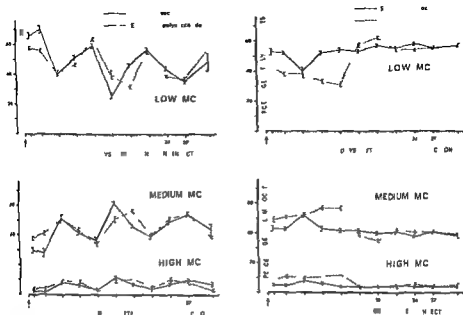


Fig 1

Percentage of lymphocytes with different mitochondrial content (MC) in blood from the carotid artery of guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi* H antigen. The arrow indicates injection of antigen. Mean \pm S.L.

The antigen injections did not influence the normal gain in body weight (about 5 g per day) or the general condition of the experimental animals. An exception was the *E coli* lipopolysaccharide which affected the condition of some animals on the first days after injection and also caused the death of a few animals.

The absolute and relative thymic weights were not significantly changed after the injection of the different antigens.

The granulocytes behaved very similarly after injection of the different antigens. In all four experiments an increased number of granulocytes occurred one day after the injection. After 3 days the number had fallen to about the normal level. The granulocytes then varied in number with maxima at 9–12 and 21–24 days and minima between. No significant difference was present between the number of granulocytes in the carotid artery and thymic vein blood.

Pertussis Vaccine

The percentage and number of small blood lymphocytes (with low MC) increased and decreased cyclically as in a previous experiment when a subcutaneous injection of the vaccine in a lower dose was given (Ernstrom & Larsson 1967a). Maxima of percentages were noted 3, 12 and 21 days after injection. The percentage of medium sized and

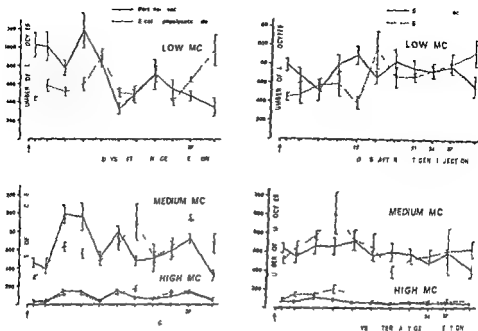


Fig 2

Number of lymphocytes per mm^3 of blood from the carotid artery in guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi* H antigen. The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC). The arrow indicates injection of antigen. Mean \pm SE.

large lymphocytes (with medium and high MC) also varied cyclically with minima when the number of small lymphocytes was maximal and *vice versa* (Fig 1). The number of lymphocytes per mm^3 showed similar cyclical variations as the percentages (Fig 2). At 31 days after the vaccine injection a decrease was recorded in all categories of lymphocytes.

The thymic veno arterial difference in number of small lymphocytes varied and 9 days after injection of the vaccine no difference was present. An import of large lymphocytes into the thymus was demonstrated with significant values at 1, 9 and 15 days after injection (Fig 3).

In the thoracic duct lymphocytes in the blood marked cyclical variations occurred in the relative frequency of the lymphocytes with low medium and high MC with the same periodicity as in the blood (Fig 7).

Lipopolysaccharide from E coli

A cyclical variation was recorded in the percentage of lymphocytes with low medium and high MC and in the number of these cells per mm^3 of blood with maximal incidence and number of small cells at

the same time is minimal incidence and number of medium sized and large cells and *vice versa* (Figs 1 and 2) The cyclicity was similar to that seen after an injection of pertussis vaccine

The thymic veno arterial difference in number of small blood lymphocytes per mm³ of blood varied and 15 and 24 days after injection no mean difference was present (Fig 1) Concurrently with this interrupted thymic export of small lymphocytes a minimum number of such lymphocytes was noted in the blood (Fig 2) At 15 and 18 days a significant thymic export of medium sized lymphocytes occurred coinciding with the finding of a maximal number of such lymphocytes in the blood A significant import of medium sized and/or large lymphocytes took place 1 12 21 and 24 days after antigen injection (Fig 4)

In the thoracic duct lymph as in the blood a marked cyclical variation was found in the percentages of small medium sized and large lymphocytes The maximal and minimal incidence of the different categories of lymphocytes as a rule occurred at the same times as in the blood (Fig 7)

Sheep Erythrocytes

The percentage of small lymphocytes was decreased after the antigen injection with a minimal value at 6 days ($p < 0.001$) Correspondingly the percentage of medium sized and large lymphocytes was raised the increase also being maximal at 11 days ($p < 0.001$) These changes were found both in thymic vein and carotid artery blood From day 9 and onwards the percentage of all categories of blood lymphocytes remained on the level of that in the normal animals (Fig 1)

The number of small lymphocytes (with low MC) was not markedly changed after injection of the antigen The number of medium sized and large lymphocytes was slightly increased 6-12 days after injection (Fig 2)

As in normal guinea pigs a thymic veno arterial difference in the number of small lymphocytes was found in the immunized animals indicating an export of small lymphocytes from the thymus One day after injection of the antigen a large export was noted but after 3 6 15 and 18 days the export was almost significantly lower than after 1 day The medium sized and large lymphocytes were more frequent in the carotid artery blood than in the thymic vein blood indicating a thymic import of such cells during immunization (Fig 5)

In the thoracic duct lymph the percentage of small lymphocytes diminished after antigen injection to a minimum value 6 days later as in the blood The incidence of medium sized and large lymphocytes were correspondingly increased At 9 days and later the composition of the lymph was about normal as regards the lymphocyte population (Fig 7)

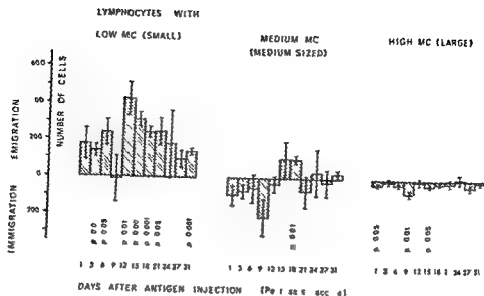


Fig 3

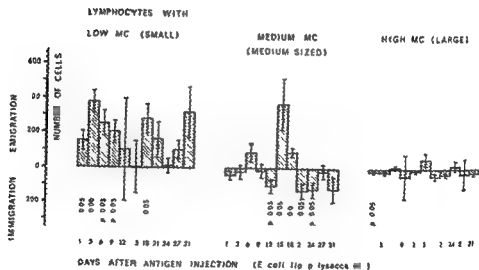


Fig 4

Figs 3-6

Thymic emigration and immigration of lymphocytes in guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E. coli* shcep ers throcytes or *S. typhi* H antigen. The bars represent the difference between the number of lymphocytes per mm³ of blood from the thymic vein and from the carotid artery. The lymphocytes are subdivided into cells with low medium and high mitochondrial content (MC). Mean \pm SE.

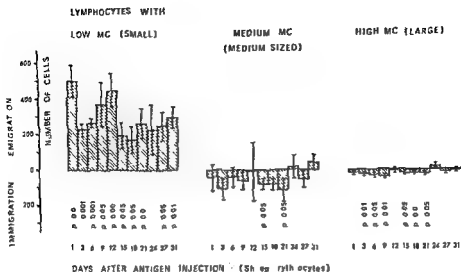


Fig 5

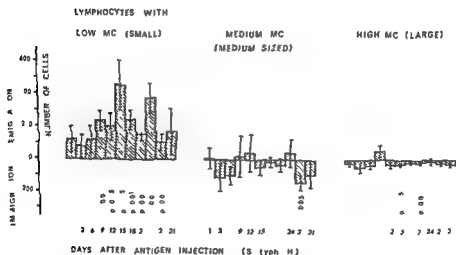


Fig 6

S typhi H

The percentage of small lymphocytes was decreased during the first 12 days after antigen injection. A corresponding increase in medium sized and large lymphocytes occurred, the increase being maximal 9 and 12 days after injection ($p < 0.001$). The changes were identical in thymic vein and carotid artery blood. From day 17 and onwards the percentage of different categories of blood lymphocytes had returned to the same level as in the normal animals (Fig 1).

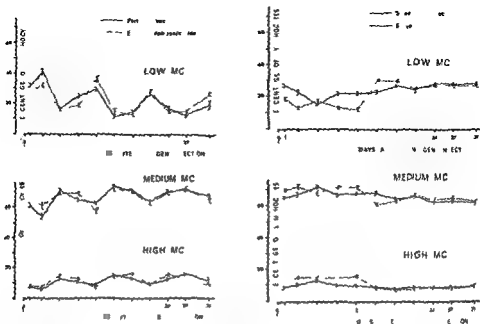


Fig 7

Percentage of lymphocytes with different mitochondrial content (MC) in thoracic duct lymph from Guinea pigs given a single injection of pertussis vaccine a lipopolysaccharide antigen from *E coli* sheep erythrocytes or *S typhi* H antigen. The arrow indicates injection of antigen. Mean \pm S.E.

During the first 12 days after administration of the typhus antigen the number of small blood lymphocytes (with low MC) was low but increased at 15 days. The number of medium sized and large cells rose monophasically with a maximum after 9 days. No cyclical variation in the number of different blood lymphocytes like that in the animals given the pertussis or coli antigen was found (Fig 2).

A significant veno arterial difference in number of small lymphocytes in the thymic vessels was observed. An import of large lymphocytes into the thymus was demonstrated after antigen injection (Fig 6).

In the thoracic duct lymph as in the blood the percentage of small lymphocytes was slightly decreased and that of medium sized and large lymphocytes correspondingly increased during the first 12 days after antigen injection (Fig 7).

DISCUSSION

The pertussis vaccine and the lipopolysaccharide from *E coli* caused the same cyclical variation in composition of the lymphocyte population in both blood and lymph. As regards the percentage of different categories of lymphocytes in blood and lymph the similarity was striking but also the number of lymphocytes per mm³ of blood showed the same cyclicity despite a relatively great variation between the total lympho-

cyte content of the blood in individual animals. In both blood and lymph the incidence of small lymphocytes was maximal about 12 and 21 days after antigen injection when the numbers of medium sized and large lymphocytes were minimal. Minimal values for small lymphocytes were found about 6 and 15 days after injection when the values for medium sized and large lymphocytes were maximal. The *S. typhi* H antigen and the sheep erythrocytes did not produce the same cyclical variation in the different categories of lymphocytes as did the pertussis and coli antigens.

The cause of the varying influence of the different antigens on the circulating lymphocyte populations is not known. The pertussis and the coli lipopolysaccharide antigens both of which caused the cyclical variation have several characteristics in common. They both contain a lipopolysaccharide antigen, are stable, remain in the body for a long time, induce production of 19S antibodies and usually no 7S antibodies. A cyclical variation recalling that described in this paper has been reported in the number of plaque-forming cells in the spleen after immunization with the lipopolysaccharide from *E. coli* (Britton & Voller 1966). The number of such cells is found to be correlated to the 19S antibody titres (Wigzell *et al.* 1966). The maximal number of splenic immunocytes was recorded 15 and 24 days after antigen injection corresponding to our maxima for medium sized and large lymphocytes. However the cyclical variation need not necessarily be caused by the antigenic properties but *e.g.* by some toxic property of the pertussis vaccine and the coli antigen.

The coli lipopolysaccharide antigen caused a significant export of medium sized lymphocytes from the thymus after 15 and 18 days (Fig. 4). A slight export at this time was also found in the animals injected with pertussis vaccine. No such export of medium sized lymphocytes was observed in the animals given the other two antigens nor was it found in earlier experiments with normal and steroid treated guinea pigs. Concurrently with this export the number of such cells in the blood was high.

The sheep erythrocytes and the *S. typhi* H antigen caused a monophasic increase in the percentage of medium sized and large lymphocytes in blood and lymph with a maximum after 6-12 days. This increase was most pronounced as regards the *S. typhi* H antigen coinciding with the appearance of agglutinating antibodies against the H antigen (Ernstrom 1965). It seems possible that these medium sized and large lymphocytes partake in the immune response perhaps as immunocytes.

Injection of pertussis vaccine resulted in a large number of small lymphocytes in the blood during the early period after injection. The animals injected with the lipopolysaccharide from *E. coli* and the *S. typhi* H antigen had low numbers of small lymphocytes in the blood during this period. At this time the thymic export of small lympho-

cytes was relatively low. This early deficiency of small lymphocytes may have been due to a stimulated secretion of adrenal steroids since previous results have shown that exogenous steroids decrease the thymic export of small lymphocytes (Ernstrom & Larsson 1967b). An activation of the adrenals caused by the antigen injection is especially plausible in the animals given the coli lipopolysaccharide which was obviously toxic in the dose administered as some animals were ill on the first day after the injection and a few of them died.

The thymic export of small lymphocytes varied after the antigen injections. As a rule a large export was accompanied by a high number of small lymphocytes in the blood and a small export by a low number. This was most obvious after injection of the lipopolysaccharide from *E. coli* which caused an interrupted thymic export of small lymphocytes after 15 and 24 days coinciding with a minimal number of such cells in the blood.

After administration of the different antigens an import of medium sized and large lymphocytes into the thymus was observed at certain intervals after injection. Such an import has recently been demonstrated during the regenerative phase after steroid induced thymic involution (Ernstrom & Larsson 1967b). This implies that the present finding may not necessarily be caused directly by the antigens or by some immune mechanism during the primary antibody response but by adrenal activation and a steroid influence on the thymus.

In all four experiments with the different antigens the granulocytes behaved similarly. Thus pronounced but temporary granulocytosis was seen 1 day after injection. About normal numbers were registered after 3 and 6 days but after 9-12 and 21-24 days the number of granulocytes in the blood was again raised. The initial granulocytosis observed 24 hours after antigen injection may have been due to adrenal activation as exogenous steroids produce pronounced granulocytosis within 6 hours (Ernstrom & Larsson 1967b). A tendency to irregular cyclical variation in granulocytes was thus observed.

The present study has demonstrated that two bacterial lipopolysaccharide antigens (from *B. pertussis* and *E. coli*) cause an almost identical cyclical variation in the circulating lymphocytes of different categories in blood and lymph and that two other antigens (sheep erythrocytes and *S. typhi* H) do not cause such a cyclical variation.

SUMMARY

Young adult guinea pigs were given a single intraperitoneal injection of one of the following antigens: pertussis vaccine, a lipopolysaccharide from *E. coli*, sheep erythrocytes, *S. typhi* H. The lymphocyte populations of carotid artery and thymic vein blood and of thoracic duct lymph were studied at different intervals after antigen injection. The lymphocytes were divided into subpopulations classified by the mito-

chondrial content of the cells (correlated to the size of the lymphocytes). The difference between the number of lymphocytes per mm³ of blood from the carotid artery and from the thymic vein indicates a thymic import or export of blood lymphocytes. The following observations were made:

1. A similar cyclical variation in composition of the circulating lymphocyte populations in blood as well as in lymph was caused by the pertussis vaccine and by the lipopolysaccharide from *E. coli*. A maximal percentage and number of small lymphocytes coincided with a minimal incidence and number of medium sized and large lymphocytes in blood and lymph about 3, 12 and 21 days after antigen injection. A minimal percentage and number of small lymphocytes and maximal of medium sized and large lymphocytes occurred after 15 and 27 days.

2. The *S. typhi* H antigen and the sheep erythrocytes did not cause the same cyclical variation in the subpopulations of the lymphocytes as did the two lipopolysaccharide antigens. The H antigen produced a monophasic increase in the incidence and number of circulating medium sized and large lymphocytes in blood and lymph with a maximum 11 days after antigen injection. The sheep erythrocytes also tended to induce a monophasic increase in medium sized and large lymphocytes.

3. An export of small lymphocytes from the thymus was found in the animals injected with the different antigens as in normal animals. As a rule a large thymic export was accompanied by a high number of small lymphocytes whereas a small export was accompanied by a low number. An import of medium sized and large lymphocytes into the thymus was observed without any obvious differences between the animals injected with the different antigens. Part of the effects observed may have been mediated by an increased secretion of thymolytic steroids from the adrenals in the antigen treated animals.

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IRRADIATION INDUCED ASYMMETRY OF THE THYMUS IN MICE

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The central role of thymus in the development of radiation induced lymphoid tumours in mice is well established (1) Spontaneous as well as virus induced thymus lymphoma may be preceded by a phase of lymphocyte depletion (2-5) With the intention to elucidate more precisely these problems concerning the radiation induced lymphoma the histological changes in the thymus after total body x ray exposure were systematically studied and analysed from the time of irradiation up to the development of thymus lymphoma

MATERIAL AND METHODS

The experiments were conducted on mice of the C3H strain since the slow development of lymphoma in this strain makes it easier to follow the development of the tumours 983 males and 297 females 25 ± 2 days old were irradiated with 500 R administered in 4 equal fractions at intervals of 4 days (6) Following the 1st irradiation the animals were killed in groups of from 17 to 53 animals during the first month every second to every third day during the second month every 10th day and thereafter monthly from the 3rd-7th month 84 males and 100 females served as untreated control animals The thymus was removed by careful dissection with intact capsul The two lobes were separated and weighed immediately on a torsion balance with the accuracy of ± 0.05 mg The lobes were fixed in Steeves fluid for histological examination

RESULTS AND DISCUSSION

The absolute weight difference of the lobes of the thymus decreased in the control animals with increasing age and decreasing total weight of the thymus (Fig 1) In the experimental animals however a successively more accentuated weight difference of the lobes of the thymus occurred in the beginning (*weight asymmetry*) The maximum difference appeared between the 23rd and 27th day after the last irradiation when the total weight of the thymus once again was increasing During this period the weight difference ranged between 5-10 mg in 29 per cent of the animals between 10-15 mg in 13 per cent and above 15 mg in 8 per cent In the control group the weight difference was under 5.1 mg in 95 per cent of the animals When the total weight of the thymus had

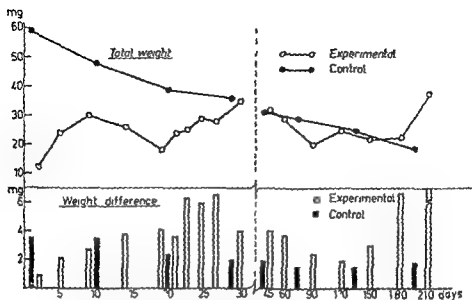


Fig 1

Mean total weight and mean weight difference of the lobes of thymus in C³H mice after 500 R of total body irradiation divided into four equal doses given every 5th day

returned to normal in the experimental animals after the first month the weight difference decreased only to increase again after 5-6 months with the appearance of an initially unilateral lymphoma

In the period immediately following the last irradiation the thymus followed histologically the earlier known pattern (7). On the second day the cortex was thin with a diffuse border toward the medulla. The number of lymphocytes had decreased and reticular cells and immature lymphoid cells dominated the picture.

On the 5th day a broader more dense partially regenerated cortex was seen and on the 9th day the first regeneration seemed to be completed. These changes appeared regularly and symmetrically in both lobes. After the 14th day the cortex became again thinner and sparse in cells and showed a diffuse border against the medulla. The number of lymphocytes was reduced and once again reticular cells became more apparent. This secondary depletion was in general symmetrically distributed in both lobes. From the 19th day the histological picture was generally more uniform within the individual lobes at the same time as the differences between the lobes of the same thymus were more marked (*early histological asymmetry*) (Fig 2).

By now the heavier lobe had recovered from the second depletion showing a cortex in regeneration or a more normal cortex. In contrast the lighter lobe was remained at the same time depleted. This depletion was occasionally further accentuated with almost complete disappearance of lymphocytes as a result (Fig 2). At this stage the cortex was

0.5mm



Fig 2

Transverse section of thymus lobes of C3H mice 70 days after irradiation
Haematoxylin and eosin

dominated by scattered reticular cells and the picture was similar to that obtained 2-3 days after irradiation with 1×550 R the cell density in the medulla being greater than that in the cortex. This second depletion was followed by a phase of regeneration which in the main agreed with the first to occur after irradiation. Immature lymphoid cells with a finer chromatin structure and basophilic cytoplasm formed successively a broad mitosis rich cortex surrounding small diffusely bordered medullary islands. Further development resulted in an apparently normal lobe of increased size and weight. In certain cases an abnormally broad cortex was seen in which medullary islands were absent or very small.

Kaplan & Brown (8) observed a similar picture of the thymus in C57Bl mice: it appeared during the regenerative phase immature cells were present together with a high mitotic activity which was interpreted as an apparent impairment of differentiation. The picture was similar to that of an early lymphoid tumour. Wasi & Block (9) also reported the finding of a temporary lymphocytic depletion in the thymus about 3 weeks after irradiation of C57Bl mice.

Once the total weight of the thymus had returned to normal after the first month the number of cases of asymmetry reduced although such asymmetry was seen throughout the rest of the experimental period (late histological asymmetry) the pictures thus exhibited were not divergent from that of the early histological asymmetry described above. In addition after 5-7 months mice were seen in which the immature lymphoid cell populations in the lighter lobe showed an increased number of mitosis and an increased number of hyperchromatic and pyknotic nuclei during the regenerative phase. In other mice these

changes were more accentuated coincident with the affected lobe now being heavier than normal and obviously the seat of a lymphoma. The transition from a regenerative phase to a lymphoma was thus very gradual. In addition to these cases of advanced bilateral lymphoma were also seen

Similar late asymmetrical depletion has previously been described as a precursory stage of spontaneous lymphoma in the thymus of 5-8 months old AKR mice (3). Asymmetrical depletion may also precede the development of virus induced thymus lymphoma in the mice (5).

In order to study more closely the further development of the asymmetrical thymus lobes biopsies were done on animals 23-29 days after the last irradiation. Under anaesthesia the thymus was exposed and a small piece of each lobe was carefully excised for histological examination whereafter the operation site was closed again. In 4 cases in which the biopsy material showed an accentuated lymphocyte depletion in one lobe and relatively normal condition in the other a reversed condition between the lobes was found at the time of sacrifice and autopsy 20-40 and 50 days later. The lobes presenting depletion in the biopsy material showed now relatively normal cell picture in three cases and immature regeneration in the fourth case. In contrast the lobes presenting normal conditions in the biopsy material now showed in three cases depletion and in one case regeneration. Serial sections of 10 lobes with accentuated depletion or regeneration showed in the majority of cases a histological picture that was relatively uniform throughout the entire lobe.

Since no degenerated lobes were seen it is probable that such alternating depletion and regeneration reflect different phases of a reversible reaction which apparently starts with an emptying of the cortex of lymphoid cells and then develops into a variable reaction pattern with successive regeneration and normalization alternating between the lobes. A phase displacement between the lobes may here mean that they have become unsynchronous which in turn is a conceivable explanation of the asymmetry which has arisen.

In order to study the possible connection between this asymmetrical thymus picture and the x irradiation animals in another experiment were irradiated with 500 R administered as a single dose. As expected fewer lymphomas were obtained (6) but also fewer and less marked asymmetries than with fractionated irradiation (in preparation). On the other hand *Kaplan & Brown* (8) found essentially the same reaction picture in the thymus following single dose or fractionated doses (same total dose 476 R) administered to C57Bl mice.

Since thigh shielding prevents the development of a secondary weight reduction in the thymus (8) and inhibits the occurrence of radiation induced lymphoma in the mouse (10) an experiment was conducted in order to ascertain the effect if any on the occurrence of the asymmetrical thymus picture. The left thigh of 24 animals was shielded during the irradiation (500 R in 4 fractions as above). When these

animals were autopsied on the 25th day the mean weight difference between the lobes of the thymus was 11.7 per cent of the total weight. In the untreated control animals the corresponding value was 6.3 per cent and in unshielded irradiated animals 27.3 per cent.

A detailed report will be presented elsewhere.

SUMMARY

Histological investigations of the histogenesis of radiation induced leukaemia in the C3H mouse have shown that an asymmetry of the lobes of the thymus as regards weight and histological pictures begins to appear during a secondary regenerative phase following irradiation. This asymmetry is observable for several months and seems to be correlated with the occurrence of unilateral lymphomas.

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the cytological estimate of tumour type and degree of differentiation. Surgical or autopsy material is available for the histopathological follow up of 304 patients. Malignant tumour was cytologically diagnosed in 373 samples (40.9 per cent) and 50 samples (5.1 per cent) were considered suspicious of malignancy. These 423 biopsy samples represent 333 patients. As regards 209 of these patients material for histopathological follow up is available. Out of the other 124 patients 31 have died the major clinical diagnosis in all cases being cancer but autopsy was not performed. The remaining 93 patients are still alive and are followed clinically.

The number of aspiration biopsies has steadily increased but because of the shorter observation period fewer histopathological comparisons can be made on the 1965 material. The number of patients per year with a diagnosis of malignancy is given in Fig. 1.

TABLE 1
Localisation of the Tumours in 209 Patients with Histological Follow Up

Site (lobe)	Histological diagnosis						Total number of patients
	Epidermoid carcinoma	Adeno carcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	Other	
Right upper	35	15	8	6	—	4	68 (33%)
Right middle	3	—	—	2	—	—	5 (2%)
Right lower	14	7	4	5	4	—	34 (16%)
Left upper	33	14	4	11	3	1	66 (32%)
Left lower	13	7	4	1	4	2	31 (15%)
Mediastinum	—	—	—	—	4	1	5 (2%)
Total	98	43	20	25	15	8	209 (100%)

The sites of the infiltrates sampled are shown in Table 1. Tumours were located in all lobes of the lungs and perihilar as well as peripheral tumours were diagnosed. The approximate diameter of the lesions was measured directly on the surgical or autopsy specimens in most instances. If a long time elapsed between aspiration biopsy and operation or autopsy or if other considerations made direct measurement unsatisfactory the diameter was estimated on the basis of radiograms with due allowance for the geometric distortion caused by X-ray divergence. In the few instances of poorly demarcated lesions measurement was not attempted. The size distribution is illustrated in Fig. 2.

Among the 209 patients from whom histopathological material was available 46 were women and 163 men. Their ages ranged from 23 to 80 years (Fig. 3).

The entire histopathological material has been re-examined by one observer with a view to obtaining uniformity in evaluation. The sections were stained according to van Gieson's method and with haematoxylin-eosin.

RESULTS

A. Tumour Incidence

A cytological diagnosis of malignant tumour was established in 192 patients. This diagnosis could be verified on the basis of the histopathological examination of surgical or autopsy material obtained from 188 patients (Table 2).

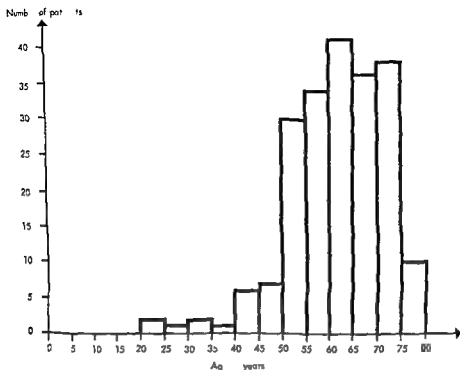


Fig 3
Age distribution of the patients

TABLE 2
Evaluation of Lung Tumours in 209 patients with Histological Follow Up on Surgical or Autopsy Specimens

Cytological diagnosis	Histological diagnosis						Total
	Malignant tumour	Tuberculosis	Non specific inflammation	Radiation pneumonitis	Hamartoma	Thymoma	
Malignant cells	188	2	1	1	-	-	192
Malignancy suspected	13	2	-	-	1	1	17
Total	201	4	1	1	1	1	209

Thus in the case of four patients (2.1 per cent) a false positive diagnosis of malignancy had been established. In two cases tuberculous changes observed in the cytological material were misinterpreted as malignant tumour. In one patient earlier irradiated for mammary carcinoma a focal infiltration in the lung was diagnosed as an adeno-

carcinoma. Examination of the histological material however revealed no signs of malignancy the lesion represented chronic inflammation and fibrosis apparently radiation induced. In a fourth patient a focal infiltration was diagnosed cytologically as a malignant tumour but after histological examination the lesion was found to be an interstitial pneumonia probably resulting from aspiration.

Among the patients in whom malignancy was suspected on the basis of the cytological findings (17 included in the histopathological follow up) 13 were found to have malignant tumours. In two patients localized tuberculous changes were observed and one had a cellular chondromatous hamartoma. In one patient a cytological sample aspirated from the mediastinum aroused the suspicion of a manifestation of malignant lymphoma or small cell cancer of oat cell type that turned out to be a thymoma.

Histopathological follow up of the 93 patients who on the basis of the cytological findings had not presented evidence or suspicion of malignancy revealed that malignant tumours were manifested in 15 cases. These 15 patients represent the false negative diagnoses. Material inadequate for cytological evaluation had been obtained from eight patients. Only inflammatory changes were seen in material aspirated from the other seven patients (Table 3).

In the total follow up material comprising 304 patients 215 malignant tumours were found. Cytological examination disclosed 87 per cent of these and gave rise to a suspicion of malignancy in a further six per cent. In 15 patients (seven per cent) with malignant tumours a cytological diagnosis was not obtained.

TABLE 3
Evaluation of 15 Patients with False Negative Diagnoses

Cytological diagnosis	Histological diagnosis					Total number of patients
	Epidermoid carcinoma	Adeno carcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	
None	2	1	—	2	3	8
Inflammation (not specified)	3	—	1	—	2	6
Tuberculosis	—	—	—	—	1	1
Total	5	1	1	2	6	15

II Type of Tumour

Cytological typing was attempted in a material comprising 282 patients with a cytological diagnosis of malignancy. The steady increase

in numbers of biopsies has made it possible to gain the experience required if the desire on the part of clinicians is to be fulfilled and more specific diagnoses are to be established. The relative number of cytologically typed tumours have increased from about 75 per cent in 1963 to 93 per cent (1964) and 98 per cent (1965). Histopathological material from 184 patients was available for a comparison with the type diagnosis made on the corresponding cytological sample.

TABLE 4
Cytological Results According to Histological Type of Tumour

Cytological diagnosis	Histological diagnosis						Total number of patients
	Epidermoid carcinoma	Adenocarcinoma	Oat cell carcinoma	Undifferentiated carcinoma	Metastasis	Other	
Epidermoid carcinoma	76 (88%)	10	4	3	—	1	94
Adenocarcinoma	4	24 (60%)	1	4	1	—	34
Oat-cell carcinoma	1	2	12 (67%)	—	—	—	15
Undifferentiated carcinoma	3	4	1	17 (71%)	—	1	26
Metastasis	2	—	—	—	12 (92%)	1	15
Total number of patients	86	40	18	24	13	3	184

A comparison between the cytological and histopathological type diagnoses is given in Table 4. Cytological typing was correct in 77 per cent of the patients. Optimal agreement (88 per cent) was obtained in the 86 cases of epidermoid tumours. As regards the somewhat smaller groups of anaplastic tumours and oat cell tumours the original cytological typing was correct in 71 per cent and 67 per cent of the cases respectively. Adenocarcinoma was most difficult to classify correctly—only 60 per cent were correctly recognized in the cytological samples. In fact 25 per cent (10 tumours) of the adenocarcinomas were cytologically typed as epidermoid tumours. Twelve of the 13 metastatic tumours were correctly recognized cytologically.

C Degree of Tumour Differentiation

Cytological material sufficient for an evaluation of the degree of tumour differentiation was obtained from 194 patients. Histopathological material to be used for a comparison was available from 122 of these patients. Any special interest in this aspect of diagnostics has not

been taken until late 1964 which may account for the relatively few cases that are available for comparison.

The degree of differentiation was denoted as high moderate low or anaplastic. If a mixed description was used for example moderately to highly differentiated the lower degree has been chosen for the purposes of this comparison.

A comparison between degrees of differentiation in the cytological and histopathological material is given in Table 5. There was good agreement in 78 per cent of the patients. The degree of differentiation was often underestimated in the cytological material obtained from moderately differentiated tumours.

TABLE 5
Cytological Results According to Degree of Tumour Differentiation

Cytological diagnosis Differentiation	Histological diagnosis Degree of differentiation				Total
	Anaplastic	Low	Moderate	High	
Anaplastic	18 (90%)	1	—	—	19 (16%)
Low	1	52 (88%)	14	2	69 (58%)
Moderate	—	6	20 (59%)	2	28 (23%)
High	—	—	—	4 (50%)	4 (3%)
Total	19 (16%)	60 (49%)	34 (28%)	8 (7%)	120 (100%)

DISCUSSION

Tumours account for about 45 per cent of all our aspiration biopsy material (Dahlgren & Nordenstrom 1966). Since an exact diagnosis of lung infiltrations can rarely be achieved on the basis of radiograms alone complementary methods are necessary to sort out the ever increasing group of patients with radiological evidence of lung infiltration. The poor prognosis associated with late detection of bronchial cancer is a spur to obtain a diagnosis as early as possible. There is every reason to have rapid and simple methods developed with a view to obtaining a diagnosis as exact as possible in order to have the adequate therapy instituted. Aspiration biopsy guided by television X-ray permits us to reach even small and relatively inaccessible tumours.

The histopathologically verified tumours in patients in this series fit in with the age and sex pattern seen in larger series of bronchial carcinomata. As has been the case in other published series (Liebow 1952; Spencer 1962) the upper lobes predominate as tumour sites. There is no distinct difference between the right and left sides. Epidermoid tumours and the undifferentiated cancers predominate in the upper lobes.

With the technique used here a cytological diagnosis can be obtained in from 80 to 90 per cent of cases of malignant intrathoracic tumours which can be detected radiologically. This result is to be seen against its background—aspiration biopsy was carried out on all types of radiologically visible lung infiltrations not solely on those suspected of malignancy.

A false positive diagnosis was made in about two per cent of the cases. Several factors have contributed to these diagnostic errors. Tuberculosis and bronchiectases particularly but also other chronic processes exert an irritating effect on epithelial cells thus producing a wide pattern of changes dependent upon the duration and degree of the irritation (Grunze 1955, Koss 1961). Degeneration or activation of epithelial cells can lead to heavy nuclear hyperchromasia with large nucleoli and vacuoles. The nuclear cytoplasm ratio can also be influenced. In routinely stained cytological materials these changes resemble those seen in malignant epithelial tumours. Only the degree of these morphological changes may distinguish an inflammatory cell reaction from a neoplastic change. Increased cell activity in inflammatory reactions can also result in a significant degree of basal cell hyperplasia. In some instances the degree and relative abundance of inflammatory epithelial changes can dominate the cytological material obtained by aspiration biopsy to such an extent that they may simulate the appearance of a malignant tumour. In the present series tuberculosis in two patients and a non specific chronic inflammation in a third together with the accompanying inflammatory changes in epithelial cells are probably the source of the erroneous diagnosis of malignancy.

Another source of error is that cells profoundly altered by radiation can be misinterpreted as malignant cells. Graham (1963) as well as others have described such cellular changes after radiation therapy. She observed that radiation induced enlargement of the cells and their nuclei with vacuolization particularly of the cytoplasm but also within the nuclei. Bizarre and sometimes multinucleated cells also appeared. These changes which may persist long after radiation therapy has been terminated can closely resemble the ones seen in cancer cells. The risk of diagnostic errors is always present if the cytologist is not informed about radiation of the region from which the sample was obtained. One of the patients in this series had received both pre and postoperative radiation therapy for mammary cancer. During a routine radiological check a focal infiltration was detected in the right upper lobe. Two attempts at aspiration biopsy were made the first was cytologically inadequate and the second gave fairly large polymorphous cells with distinct nucleoli. These were assumed to originate from an adenocarcinoma. When the resected segment was examined histopathologically it became apparent that the focal changes were radiation induced and any tumour tissue was not detected.

Russell et al (1963) have also emphasized the risk of contamination between samples as a source of false positive diagnoses. There is reason to suspect that cross contamination occurred on one occasion in the present series.

Among the cytological samples which wrongly were suspected of malignancy, later histopathological examination demonstrated the presence of a hamartoma. More recently it has become possible apparently to diagnose hamartomas cytologically (*Dahlgren* 1966). Re-examination of the original cytological sample from the patient mentioned above did in fact reveal typical hamartoma features. In this particular instance, inexperience in the cytological appearance of this type of lesion resulted in the establishment of a wrong diagnosis.

With current methods of examination it is unlikely that false positive diagnoses can be wholly avoided. This applies of course to all fields of exfoliative cytology (*Koss* 1961, *Russell et al* 1963). With improved methods the level of false positive diagnoses can probably be reduced still further.

Malignant tumours were not diagnosed in 15 patients. The principal cause of these false negative diagnoses seems to be that inadequate material had been obtained by aspiration. Either the tumour has been missed by the needle or else the tip of the needle may have entered devitalized portions of the tumour. Nearly all tumours are surrounded by a zone of chronic inflammation. If material is aspirated from this zone it is deceptively easy to be satisfied with a cytological diagnosis of inflammation.

The technique of aspiration biopsy gives the possibility of obtaining representative cells from typical and viable portions of a tumour and thereby facilitates the identification of tumour cells. There was good correlation (77 per cent) between cytological tumour type and histopathological tumour type. It is remarkable then that the correlation was only 60 per cent in the case of adenocarcinomas. Some 25 per cent of the adenocarcinomas were wrongly typed as epidermoid tumours. It was predominantly the poorly differentiated adenocarcinomas which accounted for the errors. Difficulties involved in a correct cytological recognition of adenocarcinomas has also been experienced in extensive series of exfoliative sputum cytology (*Russell et al* 1963). One reason for these difficulties may be that the staining methods used are most suitable for the recognition of epidermoid tumours. Since the poorly differentiated adenocarcinomas usually form only scanty mucus spread stains for mucus are rarely a diagnostic aid.

Any publications are not available in regards figures obtained in evaluations of degrees of tumour differentiation and comparable with our findings in similar attempts. A comparison between the degree of differentiation judged on the basis of cytological samples and findings in histopathological specimens gave agreement in about 78 per cent of the cases. There was a tendency to have the cytological degree of

differentiation placed at a rather too low level. A possible explanation of this may be that the lowest differentiated cells have a lower degree of adhesion and come to be overrepresented in aspiration biopsy samples (Coman 1944). Another source of error involved in a comparison of this type is the occasionally long interval between the performance of aspiration biopsy and the time when material for histopathological examination is obtained. During this interval a tumour can assume a lower but hardly a higher degree of differentiation.

The degree and direction of tumour differentiation can vary in different parts of a tumour. For this reason the cytological diagnosis made on isolated cells can never be as conclusive as the histopathological diagnosis based on examination of relatively large areas of a tumour.

The results obtained in these patients demonstrate that it will be possible in numerous cases to decide on the basis of cytological samples obtained by aspiration from radiologically evident lung lesions whether or not the lesion is malignant and also the type and degree of tumour differentiation. Considering the greater range of therapeutic alternatives in cases of lung tumours these aspects are of importance.

Further developments in this field of diagnostics are imperative. Even if the results to a large extent are dependent upon the personal experience of the cytopathologist, more subtle methods of fixation and staining can be expected to bring about improvement.

SUMMARY

Fine needle aspiration biopsies with the aid of television fluoroscopy were undertaken with a view to obtaining the cytological diagnosis of intrathoracic lesions. During a three year period 912 aspiration biopsies were performed on 667 patients. Malignant tumours or suspicion of malignancy were cytologically diagnosed in 333 patients. To evaluate the diagnostic reliability of this method in cases of malignant intrathoracic tumours findings in 304 patients were compared with the histopathological material obtained at operation or autopsy. The results obtained demonstrate that malignant intrathoracic tumours could be correctly diagnosed cytologically in about 87 per cent of the cases. In a further six per cent the tumour was cytologically diagnosed as malignancy suspected. In about 77 per cent of the patients it was also possible to determine correctly the tumour type and the degree of tumour differentiation.

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EXPERIMENTAL STUDIES ON BOVINE TICK BORNE FEVER

2 Differences in Virulence of Strains in Cattle and Sheep

By

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Received 20 VII 66

Foggie (1) reported that the virulence of strains of ovine tick borne fever agent he had studied could differ in sheep. Evidence which points in the same direction is to be found in the wide divergences in description by various authors of the severity of tick borne fever in sheep (7, 9, 13).

In the preceding report of this series an account was given of the isolation of 10 strains of tick borne fever agent from bovine field cases and one strain from unfed nymphs of *Ixodes ricinus* (16). Some of the strains have been studied each of them in several experimental cattle and sheep. The present paper describes the differences observed in their virulence in the respective hosts.

MATERIAL AND METHODS

The material and methods employed in this study have been mainly presented in the preceding paper of the series (16). Some additional information is given below.

Field cases from which the agent strains were isolated and the conditions. The geographical origin of each isolate has been presented previously (16). Table 1 provides some information on the field cases concerned. The findings are unsatisfactory for comparative purposes, as the time at which each cow was examined, the blood samples were taken and treatment administered varied in relation to the beginning of the reactions.

The unfed nymphs of *I. ricinus* from which J-Puke strain was isolated (15) were collected in spring 1965 from a farm pasture on which it was thought a cow had been simultaneously attacked by tick borne fever in summer 1964. The cow had exhibited depression, diminished appetite and a substantial fall in the milk yield. The maximum temperatures recorded were 41.4 and 41.5 °C and the percentages of granulocytes displaying tick borne fever bodies 35 and 41 respectively. Both cows had coughed and one had had diarrhoea. The data should be regarded with the reactions J-Puke strain had caused in experimental cattle. On this farm sheep had been kept continuously and grazed on the same pasture as cattle.

A brief account of some of the present findings was given at the IX International Congress for Microbiology in Moscow but has been published only as an abstract (14).

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Tick Borne Fever Reactions in 10 Lactating Cows in the

Strain isolated	Cow had been		Age of cow	Interval from noticed start of illness to veterinary call days	Highest recorded temp C	Proportional fall in milk yield§
	bought in recently	reared at home	y			
J34-64	×		3	0	41.2	total
Ju3-64		×	10	2	40.9	1/2
J48-64	×		2	1	41.1	1/2
J53-64	×		3	1	40.0	total
J1-65	×		2	1	41.5	total
K1-65	×		4	0	42.0	1/3
K20-65		×	2	1	41.2	1/2
T1-65	×		7	0	41.6	1/3
Jo5-65		×	3	1	41.7	1/2
Jo6-65		×	11	1	40.9	1/2

n i = no information

n f r = not fully restored

After discovery of the high virulence of K20-65 strain and a recently isolated K9-66 strain in sheep further information was obtained on the conditions on the farms of their origin. No sheep had been kept on these farms or on neighbouring farms for many years.

Serial inoculations by heterologous strains In view of an extensive immunological heterogeneity among the strains to be described in the third paper of the series (17) many animals reacted to serial inoculation of several heterologous strains. These reactions to heterologous strains in animals with previous experience of tick borne fever are partly taken into account in describing the differences in virulence of strains. This refers especially to cases in which later reactions are more severe than the earlier ones.

Criteria of virulence The following criteria were used: 1. Percentage of granulocytes displaying tick borne fever bodies and the period during which the bodies are detected; 2. Degree and duration of the temperature reaction; and 3. Length of the incubation period.

RESULTS

Degree and Duration of the Temperature Reaction

All the isolated strains invariably caused a temperature reaction when inoculated into cattle with no previous experience of tick borne fever. Sequential inoculations with heterologous strains also often induced a temperature reaction. Fig. 1 indicates the duration of reactions by different strains both in cattle with and without previous experience of the disease. No definite differences between the strains emerge from these comparisons. In regard to many strains the experiments carried out are too scanty to allow of drawing conclusions. However the impression was gained that as concerns the more extensively studied strains K20-65 tended to cause a longer fever reaction than J34-64. In Table 2 the strains are compared in respect of the highest measured temperatures they caused in older cattle without earlier experience of

Field from which Agent Strains Were Isolated 1963-1965

Depression ^a	Anorexia	Percentage of granulocytes showing bodies	Treatment	Restoration of previous milk production in days	Subsidence of fever in days
×	×	42	Tetracycline	1	1
×		20	Tetracycline	n r	n r
×		37	Tetracycline	n r	2
		24	Tetracycline	7	1
×	✓	6	Tetracycline	n r	1
×	×	38	Sulphamezathine	7	2
×	✓	75	Sulphamezathine	5	1
×	×	35	Sulphamezathine	n r	1
×	×	5	Tetracycline	n r	n r
×	×	16	Tetracycline	n r	n r

^a = temperature not recorded by the farm people^b = recorded before or at the time of the veterinary call

the disease. On the average the J34-64 and K20-65 strains resulted in a fever clearly higher than that induced by the J Puke strain. In the next paper of the series evidence will be presented that cross immunity between J05-65 strain and other tested strains appears to be very weak or absent. The mean of the highest recorded temperatures caused by this strain in 11 cattle including calves and adults was 41.2°C or within the same range as the figures given for the J34-64 and J1-65 strains.

TABLE 2

Highest Recorded Temperatures in Reactions in Cattle over 1 Year Old with no Previous Experience of Tick Borne Fever by Different Strains

	Strain of agent							
	J34-64	K20-65	J-Puke	K1-65	T1-65	J1-65	J49-64	J05-65
Number of reactions	1 ^b	4	4	1	1	1	1	1
Range °C	40.7-41.3	40.1-41.3	39.7-41.9	41.6	40.8	41.7	40.4	40.9
Mean °C	41.3	41.1	40.5					

Fig. 2 confirms this on duration of the temperature reaction in sheep. It becomes apparent that J Puke strain is more virulent in this respect than are J34-64 and J1-65 strains. It should be noted that although the K20-65 strain was tested only in sheep which had reacted earlier to other strains it matched J34-64 and J1-65 as regards duration of the induced fever. No temperature reactions were caused by J05-65, J06-65 and K1-65 strains. J05-65 strain was inoculated into two sheep and

STRAIN OF AGENT	NO TEMP. BUT TICK- BORNE FEVER BODIES	DURATION OF FEVER PERIOD DAYS							SECOND TEMP. REACTION
		1	2	3	4	5	6	7	
		● ANIMAL WITH NO EARLIER EXPERIENCE OF TICK BORNE FEVER ○ ANIMAL PREVIOUSLY INFECTED WITH ONE OR MORE HETEROLOGOUS STRAINS							
J Pu Ke					●●●●				
J 48 64				●					●
Jo 5-65			○○	○	○○○	○○○		○	○
J 34 64		○○○	●●●● ○○○	●●●●	●●●●	●●●●			●●●●
J 1-65			○○	○○○○	●	○	○		○○
J 53 64		○		○	○				
T 1 65	○○		○		●				
Ju 3 64				●					
Jo 6 65						○			○
K 1 65		○	○○	○	○○				○
K 20 65		○	○○	○○	●●	●●			●●

Fig 1

Period of elevated temperature in primary tick borne fever reactions caused by various strains in experimental cattle

Jo6 65 strain into one sheep with no earlier experience of the disease. Additionally Jo5 65 strain was inoculated into 6 sheep which had earlier reacted to other strains. K1 65 was inoculated into 3 sheep of the latter type. On the average both J PuKe and K20 65 strains induced temperatures which were definitely higher than those attributable to J34 64 and J1 65.

Occurrence of Tick Borne Fever Bodies

Fig 3 makes a comparative examination of the strains in respect of the maximum percentage of detectably infected granulocytes they caused in cattle. The lower virulence of J PuKe strain is apparent. All the 4 cattle inoculated with this strain were fully grown animals. The maximum percentages recorded in them were only 2, 2, 4 and 10 respectively. As regards the other strains, K20 65 appeared to cause higher levels of infection than three strains which were studied more extensively, Jo6 65, J34 64 and J1 65. If all the animals infected with Jo6 65 strain are considered as virtually fully susceptible, then the highest

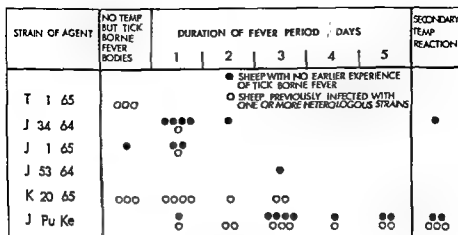


Fig 2

Period of elevated temperature in primary tick borne fever reactions caused by various strains in sheep

proportion of infected granulocytes caused by this strain tends to remain at a lower level than that attributable to J34 64 and J1 65. Attention is also drawn to the high percentage (72) of infected granulocytes caused by K1 65 strain in a heifer with no earlier experience of the disease: the data should be compared with those for the respective field case in Table 1.

Table 3 indicates that J PuKe strain is clearly inferior to other strains also as regards the duration of the period in which tick borne fever bodies were detected.

TABLE 3

Period during which Tick borne Fever Bodies Were Detected in Cattle over 1 Year Old with no Earlier Experience of Tick Borne Fever by Different Strains

	Strain of agent							
	J34-64	K20 65	J-PuKe	K1-65	T1-65	J1 65	J48-64	J53-65
Number of reactions	12	45	4	1	1	1	1	1
Range days	3-8	4-5	1-2	6	3	7	4	7
Mean days	4.4	4.5	1.8					

and 5 = on 5 and 3 cattle respectively. 1 and 5 = not sought beyond the fourth or fifth day of their occurrence.

Fig 4 presents the highest recorded levels of tick borne fever bodies in sheep by various strains. The higher virulence of the K20 65 strain than J34 64 and J1 65 is more clearly evidenced by these data than by differences in the temperature reactions given in Fig 2. Note also that K20 65 strain seems to cause levels of tick borne fever bodies in sheep

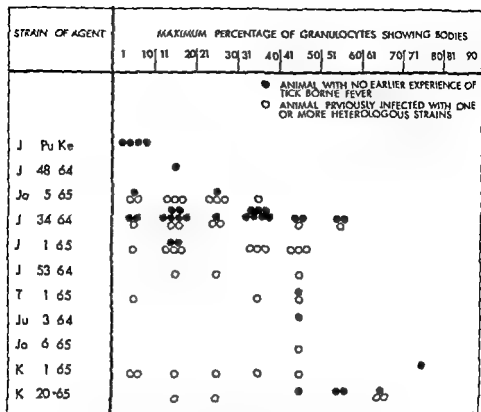


Fig 3

Highest percentage of granulocytes containing tick borne fever bodies recorded in experimental cattle reacting to inoculations by various strains (Remarks) All the inoculated cattle without previous experience of tick borne fever reacted exhibiting tick borne fever bodies. Negative inoculations into animals with previous experience of the disease are not included.

which are at least as high as those in cattle (Fig. 3). The maximum percentages of infected granulocytes caused by J PuKe strain (up to 90 per cent) were higher than those resulting from any strain in cattle (J41 64 strain 72 per cent). T1 64 strain appears to be at least as virulent in sheep as J34 64 and J1 65 if measured by the level of infected granulocytes.

The pattern displayed by different strains as regards the induced length of the period during which tick borne fever bodies were detected in sheep showed a correlation with that recognized for the maximum percentages of infected granulocytes.

Sequential Reactions by Heterologous Strains

Differences between the strains described in the foregoing are substantiated by consideration of the sequential reactions to heterologous strains in individual animals. The above comparisons suggested that

STRAIN OF AGENT	MAXIMUM PERCENTAGE OF GRANULOCYTES SHOWING BODIES												
	NO BODIES	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100		
Jo 5 65	••												
Jo 6 65	•												
T 1 65		••		•									
J 34 64		•• •	••	•									
J 1 65		••	•	•									
J 53 64				•									
K 20 65				•••	•	••		•••		•			
J Pu Ke					•• ••	• •	••	•• ••	••	• •	••	•• •	

- SHEEP WITH NO EARLIER EXPERIENCE OF TICK BORNE FEVER
- SHEEP PREVIOUSLY INFECTED WITH ONE OR MORE HETEROLOGOUS STRAINS

Fig 4

Highest percentage of granulocytes containing tick borne fever bodies recorded in experimental sheep reacting to inoculations by various strains. Negative inoculations into sheep without earlier experience of tick borne fever are included.
(Remark) Negative inoculations into sheep with previous experience of the disease are not included.

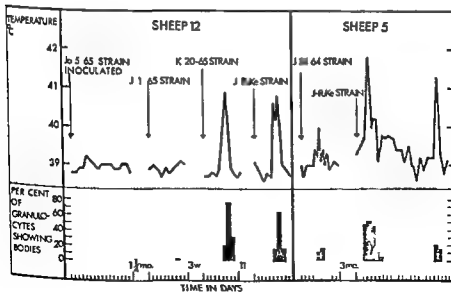


Fig 5

Comparison of tick borne fever reaction caused by inoculations of heterologous strains (5 in all) in sheep

with regard to the extent of the proportion of infected granulocytes produced in cattle four agent strains seemed to be arranged in the following order of virulence K20 65 (most virulent) J34 64 Jo5 65 and J Puke. The results compiled in Table 4 further support this contention. Although K20 65 strain was inoculated into three calves subsequent to one or two of the other strains nevertheless it induced higher levels of infected granulocytes. The inferiority of J Puke strain to Jo5 65 and J34 64 strains was also demonstrated.

TABLE 4

Comparison of 4 Agent Strains highest Percentages of Detectably Infected Granulocytes Induced in 11 Cattle Serially Inoculated by Two or Three of the Strains

Animal	Strains causing reaction their order of inoculation and the highest recorded percentage of infected granulocytes				
	1 strain	2 strain	3 strain	4 strain	5 strain
Calf 3	J34-64 (30%)	×	Jo5-65 (30%)	×	K20-65 (63%)
Calf 4	×	Jo5-65 (40%)	×	×	K20-65 (68%)
Calf 13	×	×	Jo5-65 (16%)	×	K20 65 (40%)
Cow 29	J-Puke (4%)	Jo5-65 (16%)			
Calf 26	Jo5-65 (28%)	J34 64 (52%)			
Cow 99	J-Puke (2%)	J34-64 (28%)			

× = reaction attributable to a strain not included in the comparison concerned

Differences between some strains in respect to their virulence in sheep are indicated in Fig. 5. The greater virulence of J Puke and K20 65 than J34 64 and J1 65 strains is exemplified as is the non virulence of Jo5 65 strain in sheep. (A strain K9 66 has been recently isolated from a bovine field case and found to resemble K20 65 in its virulence in sheep).

Incubation Period

The incubation period in cattle inoculated with blood obtained at about the height of the tick borne fever reaction ranged from 4 to 10 days. (Two cases in which the incubation period was prolonged obviously by partial homologous immunity are not taken into account here). This refers to all the strains examined and includes inoculations with both fresh and stored blood. No definite differences were observed in the incubation period between strains isolated from bovine field cases. However J Puke strain tended to have a longer incubation period than the other strains. This is apparent in the experiments described later in this paper.

In sheep the incubation period varied from 2 to 9 days. J Puke strain was the only strain capable of producing a clinical infection in two days. On most occasions the incubation period with this strain was 2 to 3 days. In two sheep inoculated with fresh blood containing K20 65

strain the incubation period was 3 days. Even blood infected with K20 65 strain and stored at -74°C for 1 month once induced the appearance of the first tick borne fever bodies in three days. With all other strains the incubation period was 4 days or more.

In tests with the K20 65 strain it appeared that the incubation period can apparently be prolonged under certain interesting conditions.

Four sheep were inoculated intravenously with 1 ml fresh blood obtained from a sheep at the peak of reaction to K20-65 strain. Three of the recipient sheep had experienced infection by J-Puke strain 1-3 months earlier but one of them (sheep 8) was at the time of inoculation recovering from the reaction caused by J-Puke strain. It had experienced the peak of reaction three days previously but even at the time of inoculation tick borne fever bodies were detectable in a few granulocytes. The incubation period in two sheep was three days and in the third one in which it was suspected that the inoculation had been effected subcutaneously by accident four days. In sharp contrast with these the incubation period in sheep 8 was 9 days. The reaction which developed in sheep 8 equalled that in other sheep in respect of the level of infected granulocytes. The elevation of temperature was slight in all the sheep but was hardly recognizable in sheep 8. When challenged about three weeks later sheep 9 proved to be solidly immune against K20-65 strain.

It seemed reasonable to assume that infection by J-Puke strain in sheep 8 had interfered with development of the infection by K20 65 strain. J-Puke strain as indicated previously is slightly more virulent than K20 65 strain in sheep. The question arose whether the presumed phenomenon of interference generally occurred between infections by various strains and especially whether an infection by a less virulent strain would inhibit the development of infection by a more virulent strain.

A test was carried out in six cattle over one year old to determine whether infection induced by J-Puke strain could prolong the incubation period for K20-65 strain. These strains were selected by virtue of the ease of their recognition by the difference in severity of reactions in bovine. Four cattle were inoculated with J-Puke strain and three days later two of these four and two additional cattle with K20-65 strain. The result was that the two cattle inoculated with J-Puke strain alone reacted in 7 and 10 days respectively, the two inoculated with K20 65 strain only both in 8 days and the two double infected cattle caught tick borne fever 8 days after the second inoculation. The reactions in the two double infected animals were as those in the controls inoculated with K20-65 strain. It seemed apparent that although it had been allowed a three day advantage J-Puke infection would not interfere with the development of K20-65 strain. It is an open question whether prolongation of incubation time for the K 0-65 strain would have resulted if incubation had been delayed until the appearance of the reaction to J-Puke strain.

To date it has not been possible to carry out any more systematic efforts to shed more light on the conditions of the presumed phenomenon of interference. On two occasions inoculation by a heterologous strain of animals reacting at the time to some other strain has been effected. Heifer 13 which had previously reacted to J34 64 strain and subsequently to K1 65 strain was inoculated with J1 65 strain when experiencing relapse to K1 65 strain. No reaction to J1 65 strain ensued.

However it cannot be said whether this inhibition was attributable to infection by the K1 65 strain or to developed immunity. It is suspected that the phenomenon of interference is at least partly responsible for this. In the next paper of the series data will be presented to demonstrate that immunity to neither J31 64 nor K1 65 strain alone inhibited development of the reaction by J1 65 strain.

Although no control sheep was inoculated simultaneously it was suspected that a slight prolongation of incubation time had taken place when sheep 17 was inoculated with J Puke strain at the end of clinical reaction to K20 65 strain. The incubation time for J Puke was 5 days and the following reaction was milder than the preceding one attributable to K20 65 strain.

DISCUSSION

No previous mutual comparisons have been made of strains of bovine tick borne fever agent in regard to virulence or other properties. It was indicated in the introduction that evidence of the occurrence of differences in virulence in sheep of ovine strains has been presented. A strong tendency towards variation appears to be a property common to agents of both ovine and bovine tick borne fever. That both the range and the frequency of variation of the tick borne fever agent may be very great was demonstrated on measurement of the virulence in sheep of strains treated in the present study. A range from apparent non virulence to high virulence has been presented.

Several studies, although mostly limited, have been made of the virulence of bovine tick borne fever agent in sheep or ovine agent in cattle. Neither *Hudson* (6) nor *Foggin & Allison* (3) could regularly infect sheep with the strain of the bovine agent they were investigating. *Overas & Hunsham* (10) induced mild reactions in two sheep with their agent. A similar low infectivity of ovine strains for cattle has been recorded (1). However *Hudson* (6) did not mention any difficulties in infecting cattle with a Scottish ovine strain although he did not describe these reactions in detail. Thus a higher virulence for the natural host of the strains studied has been a general observation. This has led to the postulation of considerable differences between strains associated with bovine or ovine disease under natural conditions (2-3). It has further been considered probable that in a limited area there could only be present either the ovine or the bovine strain of the tick borne fever agent dependent upon whether sheep or cattle constitute the major grazing population (3-19).

Higher virulence in the natural host cattle was also the property of most Finnish strains isolated from bovine field cases. Nonetheless two strains (K20 65 and K9 66) appeared to possess about the same degree of virulence in sheep and in cattle. The occurrence of this type of strain has not been described earlier. It is interesting that no sheep had been

kept on the farms of origin of these strains. This finding is thought to indicate that the difference between the variants of the tick borne fever agent which induce natural disease in sheep and those affecting cattle need not in general be so strict as has been thought earlier. The occurrence of intermediary forms may be relatively frequent. Absence of sheep on the farms on which K20 6a and K9 66 strains originated suggests that development of a strain of high virulence in sheep would not necessarily presuppose an adaptation to sheep but could perhaps also be the results of random variation.

Throughout Finland cattle far outnumber sheep (15). According to the theory that either an ovine or a bovine variant of the agents achieves dominance in the area and excludes the other (3, 19) a build up of sheep strains would be improbable in Finland. Isolation from the ticks of J Pike strain which was highly virulent in sheep and of only low virulence in cattle was therefore surprising. Even more surprising was the fact that on the same pasture there was obviously present another strain virulent in cattle (see Material and Methods). From the present experimental results it has emerged that an animal may within a short time be infected with several heterologous strains. In the following paper (17) evidence will be given that two heterologous strains may occur even simultaneously in one animal. Thus there appears to be no theoretical objection to the possible existence of two clearly different strains on a pasture.

It is considered highly improbable that the conspicuous prolongation of the incubation time for K20 6a strain in sheep III could have been effected by specific cross immunity induced by heterologous strains. No indication had appeared of the operation of such a mechanism in comparable tests with several sheep. (The immunological difference of the strains concerned will be well documented by the results presented in the next paper of the series (17)). If it is not specific immunity what then is the nature of the enhanced resistance? Although to date the subject has not been studied to an extent which justifies drawing any conclusions speculation in this connection already appears worthwhile. It could be possible that this phenomenon is effected by an interference mechanism not too remote from that described for viruses. There are reports that infections by organisms bearing similarities to tick borne fever agent (18) by some rickettsiae induce the formation of interferon like substances (19). It has also been reported that infection by *Brucella abortus* produces interferon (20). The point at issue is whether interferon like substances or some other substances like endogenous pyrogen (tick borne fever is characterized by high fever and destruction of granulocytes) could in some way mediate in the inhibition of intracellular multiplication of these bacteria like organisms? The first indication that interferon might in fact do so has been provided by Hanna Merigan & Jawet (4) who demonstrated that virus induced interferon inhibited the multiplication of a TRIC agent

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SEROLOGICAL TYPING OF *STAPHYLOCOCCUS AUREUS*

7 Technical Aspects

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Several new type agglutinogens have been described in *Staphylococcus aureus* (3-4, 5, 6, 7, 8). The number of type strains has also been extended being now 18. All type strains have been examined by slide agglutination in absorbed rabbit immune sera, the so called factor sera (13). Portions of the factor sera have been further absorbed with one strain each and thereafter tested again for residual agglutinins. Similar absorptions were carried out with the whole immune sera and new factor sera thus produced were in turn absorbed with each type strain as above until completely exhausted. Thereby all 18 type strains were characterized by their agglutinogen pattern, their absorbing capacity and their ability to elicit agglutinins in rabbits.

In the course of this work it was repeatedly felt that parts of the typing technique required a critical re-examination, the result of which has been presented here. Included is also a summary up of technical modifications reported in earlier articles (5, 6).

MATERIALS AND METHODS

Strains and cultivations. The 18 serological type strains (3, 5, 8, 13) were grown on nutrient agar slants and mannitol salt agar (Difco) plates overnight at 37°C. For demonstration of the *h* and 760 antigens mannitol salt agar plates were incubated overnight at 25°C.

Immunization, agglutination and absorption procedures were the same as before (13) when not described separately under Experiments.

EXPERIMENTS AND RESULTS

Among technical problems met with in serological typing of *Staph aureus* four are of major importance. False clumping of the bacteria, normal agglutinins against staphylococci in rabbits, varying antibody response and blocking of antigens.

False Clumping

The term false clumping is used here to designate an aggregation of bacterial cells not caused by antibody as opposed to true agglutination.

Most colonies of *Staph aureus* are of the smooth type. In suspension however the organisms frequently show a tendency to aggregate a troublesome property which in some studies has rendered many strains non typable. Moreover for grounds to be presented below there is reason to believe that false clumping has sometimes been misinterpreted as true agglutination.

Slide agglutination of staphylococci is performed with colonies taken up with a loop from the agar surface. Bacteria deriving from different areas of the agar occasionally show different degrees of stability in suspension.

This may require the inclusion of a control suspension with bacteria taken from the same area in every testing of the strain. Alternatively a suspension of the organisms can be prepared beforehand for use in all tests. The latter procedure will cause further dilution of factor serum and should as a rule be avoided. This problem may however be considered of minor importance provided its possible occurrence is borne in mind.

Every microbiologist will surely be familiar with the clumping of *Staph aureus* in the presence of fibrinogen and it is hardly necessary to warn against the use of plasma instead of serum in serological typing of this bacterium. However this brings up the point of the stability of staphylococci in suspension being greatly influenced by the composition of the suspension fluid. A factor serum is an immune serum which has been absorbed with organisms harvested from nutrient agar. In addition to specific antibodies and normal serum components the factor serum contains substances from the culture medium as well as materials extracted from the bacteria. It is therefore obvious that saline is by no means a satisfactory suspension fluid for the control of false clumping. This is illustrated by an example in Table 1.

TABLE 1
False Clumping Revealed by the Use of an Absorbed Serum as a Control

Strain	n serum	Agglutination in saline	n serum absorbed with strain F21
F21	++	—	++

Grade + to +++ strength of agglutination — No agglutination

It is seen from Table 1 that strain F21 was agglutinated by the n serum the saline control being negative. However the nature of the clumping gave rise to suspicion of false clumping. The n serum was therefore absorbed with strain F21. The absorption had no influence on the clumping which accordingly was false although a stable suspension was obtained with saline.

Consequently we recommend suspension of the bacteria as a con-

trol in a rabbit serum diluted 1 to 10 of which the agglutinins have been exhausted by absorption.

As mentioned above it was the character of the clumping, that gave rise to suspicion of false clumping. It may be of practical importance to be familiar with some of the features that distinguish false clumping from true agglutination. In false clumping the organisms aggregate in the centre of the drop and have a tendency to sink below its surface. In true agglutination on the other hand the organisms become oriented peripherically in the drop and tend to float. Moreover, false clumping reaches a maximum very soon whereas true agglutination shows a gradual increase in strength on continued rocking of the slide. When both occur concurrently the above mentioned features may provide evidence for the presence of a true agglutination masked by a false clumping. In a strain like F-21 which potentially is spontaneously agglutinable a weak agglutination is often augmented by a superimposed false clumping triggered by the agglutination which may then be misinterpreted as a strong reaction.

Several means have been proposed to avoid the false clumping: the addition of anti-formin, the autoclaving of the organisms in increased salt concentration (mannitol salt agar) and the use of fresh young nutrient agar cultures. The two first procedures will alter the milieu in which the agglutination takes place and the sensitivity of the test will then have to be checked for each factor serum. Autoclaving destroys some agglutinogens and cultivation on mannitol salt agar may influence the synthesis of antigens. In this laboratory we have mostly resorted to the use of fresh 5 hour old nutrient agar cultures which are more stable in suspensions than older cultures. As shown by Oeding (15) these young cultures may give stronger agglutination but with most factor sera they give the same results as fresh 24 hours cultures. If a 5 hour culture shows false clumping, it is transferred to a new culture and tested again. After one or more transfers a stable suspension is usually obtained.

Agglutinins against Staph aureus in Pre Immune Rabbit Sera

Most of the pre immune sera of our albino rabbits agglutinate *Staph aureus* weakly. In the standard procedure (15) the serum is tested in dilution 1:10 for agglutinins against the strain to be used for immunization. If there is little or no agglutination the immunization is started. This gives rise to two questions: Is it sufficient to test the serum with the immunizing strain only? If weak agglutinins are demonstrated can we be sure that the agglutinins are removed by the later absorption for preparation of factor sera and will thus not interfere with the typing?

To examine this question 40 sera from non immunized rabbits were tested in dilution 1:10 for agglutinins against the 18 *Staph aureus* type strains. All of the sera agglutinated several strains. Some strains

Wood 46 Cowan II 670 5687 and 6376 were agglutinated by a few sera only or not at all. Strain Cowan I on the other hand was agglutinated by all sera. The agglutinin patterns indicated the presence of several different agglutinins. No attempts were made to differentiate or identify the individual agglutinins.

With regard to the first question it is clear from these investigations that pre immune sera must be tested with all type strains.

Still more important is the next question. Will the normal agglutinins be removed by the absorptions made later for preparation of the factor sera? This was examined by testing the type strains for their capacity to absorb normal agglutinins. Five sera representing the different agglutinin patterns were pooled and diluted 1:2. Portions of the pool were absorbed with one type strain each and tested for residual agglutinins. The absorbing capacity appeared to vary considerably from one strain to another. While the standard absorption dose of strain Cowan I organisms absorbed all agglutinins, three times this dose of strain Wood 46 showed almost no absorption. When the standard dose of organisms was used eight strains namely strains 28 Wood 46 17A Cowan II Cowan III 1015 830 and 5687 showed inadequate absorption of normal agglutinins.

Since little is known about the nature and distribution of normal agglutinins in different colonies and strains of rabbits, no general rule can be given as to which strains should be included in the absorptions for preparation of factor sera. Before inoculation the serum should therefore be tested in dilution 1:10 with all type strains. Thereafter the absorbing capacity for possible agglutinins is tested with the strain or strains to be used later for absorbing the immune serum.

TABLE 2

Agglutination of Type Strains in a Pre Immune Serum Before and after Absorption

Strain	Agglutination in pre immune		tut (1:10)
	not abs	abs	with st. 1
1503	+	—	
99	++	—	
174	+++	—	
Cowan I	++++	—	
830	+	—	
Other type strains	—	—	

Note: see Table 1

An example illustrates this (Table 2). It appears from Table 2 that six strains were agglutinated by the pre immune rabbit serum diluted 1:10. The resulting immune serum is intended for preparation of factor serum which is made from antiserum to strain 3647 by absorbing

with strain F21. The absorbing capacity of strain F21 for normal agglutinins is therefore tested. It is seen from Table 2 that all agglutinins were removed by this absorption. We can conclude that this rabbit can be used for immunization with strain 3647 for preparation of factor α serum.

It was commonly observed in these experiments that several strains showed false clumping when normal sera were used in a concentration greater than 1:10. In some sera strain Cowan I also showed false clumping in dilution 1:10. It is likely that false clumping, such as this is due to special physico-chemical properties of the serum. In serological typing of staphylococci we must therefore advise against using factor sera more concentrated than 1:10. The latter observation also adds further support to our opinion expressed above that a saline suspension is not a satisfactory control.

TABLE 3
Antibody Composition of Two Rabbit Sera (h727 and h798) after Immunization with Strain 3647

Antibodies against	Serum h727	Serum h798
a_1	++	—
a	++	—
a_2	++	+
a_3	+++	+++
σ	++	++
b_1	+	+++
c_1	(+)	+++

Code: (+) to +++ denote content of agglutinins. — Agglutinins not demonstrated.

Immunization Problems

Rabbits vary considerably in antibody response to staphylococcal antigens. Some rabbits are generally poor antibody producers, others produce one antibody abundantly but little of another. The latter is illustrated in Table 3. Two rabbits were immunized with strain 3647, a strain frequently employed for immunization. The rabbits were given parallel injections from the same batch of organisms. It is seen from the table that both rabbits produced potent agglutinins against antigens a_1 and a , the antibody composition varying widely in other respects. Agglutinins to a_1 and a could not be demonstrated in serum h728 which on the other hand had strong b_1 and c_1 agglutinins. In order to save time several rabbits should therefore be immunized simultaneously. The complete antibody composition of an immune serum will sometimes be of interest because a serum which cannot be used for the planned factor serum may appear to be suitable for preparation of another.

The results of an immunization depend primarily on the selection

of the right strain. The presence of a strong agglutinin in a strain is no guarantee of a successful immunization. A strain which is not agglutinated at all may appear to be the best one for production of the respective antibody and it appears that the choice of strain is based on practical experience.

If there is a poor antibody response after one series of injections further immunization will often be of no use. After a successful immunization maximal amounts of serum should be withdrawn since a later booster injection may produce different agglutinin patterns.

Blocked Antigens

It is the author's opinion that the greatest difficulty in serological classification of staphylococci is the common occurrence of blocked agglutinogens. Antigen blocking means that under certain conditions antigenic groupings are not free to react with the agglutinin. The cause of this is not known, most likely it is steric hindrance. The same type of blocking is met with in strains of *F. coli* and *Salmonella* (blocking of somatic antigens by H and V antigens).

TABLE 4
Methods Used for Unmasking of Blocked Antigens in Staphylococci

-
- | | |
|---|--|
| 1 | Treatment with trypsin (14) |
| 2 | Autoclaving (1, 14, 16) |
| 3 | Fresh 5 hour old nutrient agar cultures (15) |
| 4 | Mannitol salt agar cultures 37° C (6) |
| 5 | Mannitol salt agar cultures 25° C (6) |
-

Many methods have been tried for unmasking of blocked antigens. A list of methods used in typing of staphylococci has been presented in Table 4. Treatment with trypsin (14) is time-consuming, destroys protein antigens and may cause false clumping. Oeding (14) used both autoclaved and live bacteria in his early work, since autoclaving revealed antigens which could not be demonstrated by means of live organisms. Later (15) he found that autoclaving could be omitted when he used fresh young cultures, preferably 5 hours old. When several new staphylococcal antigens were discovered (6) it soon became clear to the author that 5 hour cultures could by no means replace autoclaved bacteria. Autoclaving was not however a very sensitive method for the revelation of blocked antigens. In a search for newer methods mannitol salt agar cultures of the bacteria grown at 37° and 25° C (6) were found to give stronger reactions than autoclaved organisms. Moreover the method was much simpler.

Agglutinin blocking, as met with in staphylococci, is illustrated in Tables 5 and 6. It is seen in Table 5 that a live nutrient agar culture of strain 3647 was not agglutinated by a serum in contrast to autoclaved

with strain F-21. The absorbency capacity of strain F-21 for normal agglutinins is therefore tested. It is seen from Table 2 that all agglutinins were removed by this absorption. We can conclude that this rabbit can be used for immunization with strain 3647 for preparation of factor a serum.

It was commonly observed in these experiments that several strains showed false clumping when normal sera were used in a concentration greater than 1:10. In some sera strain Cowan I also showed false clumping in dilution 1:10. It is likely that false clumping such as this is due to special physico-chemical properties of the serum. In serological typing of staphylococci we must therefore advise against using factor sera more concentrated than 1:10. The latter observation also adds further support to our opinion expressed above that a saline suspension is not a satisfactory control.

TABLE 3
Antibody Composition of Two Rabbit Sera (k 727 and k 728) after Immunization with Strain 3647

Antibodies against	Serum k727	Serum k728
a_1	++	---
a	++	---
a_2	++	+
a_3	+++	+++
a	++	++
b_1	+	+++
c_1	(+)	+++

Code (+) to +++ denote content of agglutinins --- Agglutinins not demonstrated

Immunization Problems

Rabbits vary considerably in antibody response to staphylococcal antigens. Some rabbits are generally poor antibody producers, others produce one antibody abundantly but little of another. The latter is illustrated in Table 3. Two rabbits were immunized with strain 3647, a strain frequently employed for immunization. The rabbits were given parallel injections from the same batch of organisms. It is seen from the table that both rabbits produced potent agglutinins against antigens a_1 and a , the antibody composition varying widely in other respects. Agglutinins to a_1 and a could not be demonstrated in serum k728 which on the other hand had strong b_1 and c_1 agglutinins. In order to save time several rabbits should therefore be immunized simultaneously. The complete antibody composition of an immune serum will some times be of interest because a serum which cannot be used for the planned factor serum may appear to be suitable for preparation of another.

The results of an immunization depend primarily on the selection

of the right strain. The presence of a strong agglutinogen in a strain is no guarantee of a successful immunization. A strain which is not agglutinated at all may appear to be the best one for production of the respective antibody and it appears that the choice of strain is based on practical experience.

If there is a poor antibody response after one series of injections further immunization will often be of no use. After a successful immunization maximal amounts of serum should be withdrawn since a later booster injection may produce different agglutination patterns.

Blocked Antigens

It is the author's opinion that the greatest difficulty in serological classification of staphylococci is the common occurrence of blocked agglutinogens. Antigen blocking means that under certain conditions antigenic groupings are not free to react with the agglutinin. The cause of this is not known, most likely it is steric hindrance. The same type of blocking is met with in strains of *E. coli* and salmonella (blocking of somatic antigens by *H* and *V*₁ antigens).

TABLE 4

Methods Used for Unmasking of Blocked Antigens in Staphylococci

-
- | | |
|---|--|
| 1 | Treatment with trypsin (14) |
| 2 | Autoclaving (14, 16) |
| 3 | Fresh 8 hour old nutrient agar cultures (15) |
| 4 | Mannitol salt agar cultures 37° C (6) |
| 5 | Mannitol salt agar cultures 25° C (6) |
-

Many methods have been tried for unmasking of blocked antigens. A list of methods used in typing of staphylococci has been presented in Table 4. Treatment with trypsin (14) is time-consuming, destroys protein antigens and may cause false clumping. Oeding (14) used both autoclaved and live bacteria in his early work, since autoclaving revealed antigens which could not be demonstrated by means of live organisms. Later (15) he found that autoclaving could be omitted if he used fresh young cultures preferably 5 hours old. When several staphylococcal antigens were discovered (6) it soon became clear to the author that 5 hour cultures could by no means replace autoclaved bacteria. Autoclaving was not however a very sensitive method for the revelation of blocked antigens. In a search for newer methods, salt agar cultures of the bacteria grown at 37° and 25° C were found to give stronger reactions than autoclaved organisms, and the method was much simpler.

Agglutinogen blocking is met with in staphylococci (Tables 5 and 6). It is seen in Table 5 that a live nutrient strain 3647 was not agglutinated by a serum in contrast

development of a set of type strains. Our 18 type strains have been mapped antigenically by agglutinogen patterns, absorbing capacity, and agglutinin production in rabbits. We have introduced an additional culture medium, mannitol salt agar, and in some instances the bacteria are grown both at 37° and 25° C. In this way the method has been rendered considerably more sensitive by means of a simple and safe modification. As might be expected, the absorption experiments revealed some weak agglutinogens which could not be demonstrated by agglutination.

The main problem is undoubtedly the commonly occurring blocking of antigens. The *h* antigen, for instance, cannot be detected by the introduction of a tube test by a prolonged incubation or by a finer reading, since it is blocked in most strains grown on nutrient agar.

Cultivation on mannitol salt agar was found to be superior to auto-claving in unmasking of the blocking. The mannitol salt medium differs from nutrient agar in the high sodium chloride concentration (8.5 per cent) and the mannitol content of the former. Mannitol is oxidized by *Staph. aureus* resulting in acid formation. As reported in an earlier article (6) the presence of mannitol and the low pH have no influence on the typing, and the unmasking effect was found to be due to the high salt concentration. We have, however, preferred to employ mannitol salt agar, since this medium is commonly used in laboratories.

The nature of the blocking of antigens in staphylococci has not been established. On the basis of the results of trypsinization and autoclaving, of the bacteria Oeding (14) proposed a model for the antigenic build up of a staphylococcus: an outer protein and an inner polysaccharide layer. How blocked antigens are revealed by cultivation at a high salt concentration and/or at low temperature is not clear. If we presume an outer protein layer, it is likely that the salt and the low temperature inhibit the synthesis of these proteins, thus exposing the polysaccharide layer.

On the basis of our present experience with the type strains, the following procedure is proposed for demonstration of agglutinogens. Bacteria grown on mannitol salt agar for 18 hours at 25° C. are used for testing in factor sera *a*, *c*₁, *h*, *l*₁, and 670-1. For use with the remaining sera, the bacteria should be cultivated on nutrient agar for 18 hours at 37° C. The results of typing of many other strains must be awaited before more definite directions can be given.

The staphylococcal agglutinogens known at present have been identified by examining absorbed immune sera for agglutinins against the 18 type strains. The number of immune sera examined, as well as the number of strains employed for absorption, are limited, although the strains are selected from many others tested. If other strains and more rabbits are used for immunization, new agglutinogens will most probably be found. Neither can the possibility be excluded that our present sera contain other agglutinins which might be detected if other strains

were introduced for absorption. However, if further progress is to be made in the serological classification of staphylococci, it is at present more important to standardize the technique and to get more experience and information about the value of the new factor sera in practical typing work.

We are inclined to emphasize that those who adopt our method should be familiar with the agglutination patterns of each type strain and check all factor sera as indicated under "Summary". Any alteration may indicate interference by other agglutinating factors and should be cleared up to avoid conflicting results. An exchange of strains between laboratories for comparison of typing results is to be recommended.

SUMMARY AND CONCLUSIONS

The technique for serological typing of *Staph. aureus* has been critically examined. The following general technical procedures are recommended as a supplement to Oeding's (15) standard method.

1. *Immunization* a) Pre-immune sera diluted 1:10 should be tested with all type strains.

b) The strains to be employed in later absorption of the immune serum should be capable of removing all agglutinins in the pre-immune serum.

c) Booster injections should be avoided.

2. *Agglutination* a) As a control for false clumping, saline is replaced by an exhausted immune or pre-immune rabbit serum diluted 1:10.

b) Agglutination is carried out with bacteria grown for 18 hours on nutrient agar at 37°C and on mannitol salt agar at 25°C.

Further details are given above.

c) Before use, a factor serum should be checked with all type strains.

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AN IN VITRO STUDY OF THE ANTIBIOTIC SENSITIVITY OF *FLAVOBACTERIUM MENINGOSEPTICUM*

By

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Flavobacterium meningosepticum is a gram negative rod first described by Elizabeth O. King in 1959. Since then six serologically different types termed A-F have been found. The microorganism was isolated from blood and spinal fluid of premature infants with severe meningitis. In total reports of 63 such cases have been published (Brody *et al* 1958, Vandepitte *et al* 1958, Cabrera & Davis 1961, George *et al* 1961, Seligmann *et al* 1963, Sugathadasa & Arsecularatne 1963, Vandepitte *et al* 1965, Watson *et al* 1966). In addition the microorganism has been isolated postoperatively from the blood of adult individuals. Prognoses in these cases were fair (Olsen *et al* 1965).

In the individual studies the antibiotic sensitivity has been found to vary. Vandepitte *et al* (1965) found sensitivity to streptomycin and tetracycline and resistance to all other antibiotics. All other investigators have found sensitivity to erythromycin and chloramphenicol and resistance to penicillin and polymyxin. In addition King (1959) found resistance to tetracycline, streptomycin, neomycin and bacitracin while others have found sensitivity to tetracycline and streptomycin (Vandepitte *et al* 1958, Sugathadasa & Arsecularatne 1963). Watson *et al* (1966) found sensitivity to neomycin and resistance to streptomycin, tetracycline and ampicillin. No data concerning the method used by the last mentioned authors. The other investigators mentioned used an agar diffusion method.

Although sensitivity to one or several antibiotics has been found in all cases therapeutic results have been poor. Among 51 well documented cases the outcome was fatal in 38 and absence of sequelae was seen only in four patients.

The object of this study has been on the basis of *in vitro* experiments to form an idea of the antibiotic treatment which could be expected to be most effective against infections caused by *Flavobacterium meningosepticum*.

METHODS

1 Estimation of 50 per cent Inhibitory Concentration (IC 50)

Two two fold dilution series of an antibiotic dissolved in broth were produced. Each tube contained 0.5 ml and the first tubes generally holding 1000 µg/ml of antibiotic. The tubes were inoculated by means of a platinum loop and incubated for two days at 35 °C upon which blood plates were inoculated using matter from all tubes without visible growth in order to determine whether the effect was of a bactericidal or bacteriostatic nature.

From the number of tubes with growth a titre was estimated according to Karber's method and the IC 50 was calculated by the equation

$$IC_{50} \approx \frac{1000}{2^{10-T}} \sim 2^T$$

where T is the estimated Karber titre when the initial tube is termed 10 and contains 1000 µg/ml antibiotic.

The inoculum estimated in all tests was 10⁴-10⁵ per tube. The size of the inoculum was estimated by production of a ten fold dilution series of the bacterial culture followed by inoculation of 0.1 ml onto blood agar plates.

Among a total of 314 double series 236 gave the same end point for the two series in 53 series the difference was one tube and in three there was a difference of two tubes. If a double series is considered as a double estimation of the end point the standard deviation of the estimation can be calculated to 0.23 (*f* = 314).

2 Combination of Two Antibiotics

a Combination of two antibiotics with IC 50 > 40 µg/ml. If the antibiotics to be studied in pairs were termed A, B and C the tubes should be placed according to the following scheme (Carroll & Waterworth 1962)

AA	AB	AC
	BB	BC
		CC

In continuous serum concentrations above 20 µg/ml of an antibiotic cannot be expected a tube of type AB was made to contain 20 µg/ml of A + 20 µg/ml of B. Tubes of type AA contained 40 µg/ml of A. The tubes were inoculated and incubated for two days at 35 °C. In tubes without growth a potentiating effect of the two antibiotics must be present. Such combinations were further studied using the method described later under c.

b Combination of antibiotics with IC 50 > 40 µg/ml and an antibiotic with IC 50 < 40 µg/ml. A number of two fold dilution series were produced using an antibiotic A with an IC 50 < 40 µg/ml. One double series contained antibiotic A exclusively but 20 µg/ml of all antibiotics with an IC 50 > 40 µg/ml were added to the remaining double series. Subsequently they were inoculated and incubated at 35 °C for two days. If upon addition of an antibiotic B a fall in the titre was found synergism between A and B must be present. An increase in the titre indicates antagonism between A and B.

A fall of one log unit in the titre indicates that 20 µg/ml of antibiotic B added replaces up to 50 per cent of IC 50 of antibiotic A. A fall in the titre exceeding one log unit indicates the presence of a potentiating effect.

All antibiotic combinations showing a fall in the titre after incubation for two days were further studied using the method described under c.

c Combination of two antibiotics with IC 50 < 40 µg/ml. Of the two antibiotics A and B that were to be combined a two fold dilution series was produced. Next 64 tubes were placed in a check pattern. Tubes recorded in column furthest to the right contained antibiotic A exclusively in a two fold dilution series and those recorded in the lowermost horizontal row contained antibiotic B exclusively in a two fold dilution series. The remaining tubes contained A and B in all concentrations that could be produced by combinations of the two dilution series. The tubes were inoculated and incubated for two days at 35 °C upon which the number of tubes with visible growth was recorded.

ERYTHROMYCIN

40 20 10 5 2.5 1.25 0.63 0.00

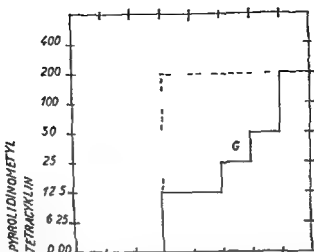


Fig 1

A diagram depicting results obtained by a combination of erythromycin/pyrolidino methyl tetracycline using method "c". The figures represent concentrations in $\mu\text{g/ml}$ of the two antibiotics. Growth appeared in the tubes below and to the right of the polygonal line. Tube G contains $1/8$ of the least inhibitory concentration of both antibiotics; thus a potentiating effect is present.

Using suitable initial concentrations, zones without growth will be present to the left and in the upper part of the system as concentrations at these sites should exceed the lowest inhibitory concentrations of the two antibiotics. In the following figures these zones are seen to the left and above the dotted line. An appearance of growth in these zones indicates an antagonism between the two antibiotics (Fig 2).

If a tube without growth is found below and to the right of the two zones synergism is present (Fig 1). If the concentrations of the two antibiotics are expressed in fractions of the lowest inhibitory concentration, a measurement of the synergistic effect will be obtained in the sum of these fractions in the tube without growth in which this sum is smallest.

A definite potentiating effect will be present if a tube without growth is found in which both antibiotics have been diluted at least two squares (four fold) from their lowest inhibitory concentrations.

In order to obtain an expression of the combination effect evaluated on the basis of a bactericidal point of view, all tubes without visible growth were used for inoculation onto blood agar plates.

Strains Employed

Thirteen serologically different strains were used. They have all been isolated from premature infants with meningitis.

A King no	14	ATCC 13133
B King no	499	ATCC 13144
C King no	3375	ATCC 13155
D King no	6915	
E King no	8788	
F King no	8707	

VANCOMYCIN

400 200 100 50 25 12.5 6.25 3.13 0.00

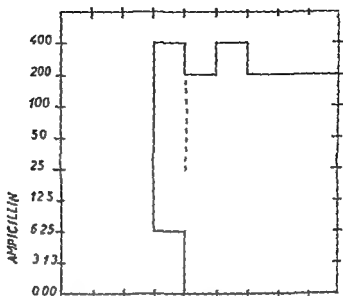


Fig. 2

A diagram depicting results obtained by a combination of vancomycin/ampicillin. Antagonism exists as growth appeared in several tubes in zones where the lowest inhibitory concentration of the one antibiotic was exceeded.

In addition the following strains were studied which were all isolated from the Department of Thoracic Surgery, Odense City and County Hospital.

523 isolated from blood of a patient (J no 267/64)

6697 isolated from blood of a patient (J no 456/64)

6846 isolated from blood of a patient (J no 390/64)

T 396 isolated from a bronchoscopic suction apparatus

L 5 isolated from the air in an operating theatre

The biochemical properties of the strains were described by Kim (1959), Olsen *et al* (1965) and Olsen (1966).

RESULTS

The 50 per cent inhibitory concentrations (IC 50) found are recorded in Table 1. The log IC 50 represents mean values. N indicates the number of tests from which the mean values were calculated. The IC 50 were calculated on the basis of the mean values and recorded in $\mu\text{g/ml}$.

The following antibiotics were found to be bactericidal: Vancomycin, penicillin, ampicillin and streptomycin. The remaining ones were found to have a bacteriostatic effect. In the novobiocin tests numerous surviving colonies were found even at high concentrations whilst the other bacteriostatic antibiotics produced a bactericidal effect in concentrations two to four times that of the inhibitory concentration.

TABLE 1a
Determination of Sensitivity of H. Flavobacterium meningosepticum Strains

	Erythromycin lactobionate		Novobiocin sodium (Albamin)		Vancomycin hydrochloride		Fusidin		Benzyl penicillin sodium	
	IC ₅₀ µg/ml	N	IC ₅₀ µg/ml	log ₁₀ IC ₅₀	IC ₅₀ µg/ml	log ₁₀ IC ₅₀	IC ₅₀ µg/ml	log ₁₀ IC ₅₀	IC ₅₀ µg/ml	log ₁₀ IC ₅₀
King A	11	35	1	25	55	28	15	39	34	51
King B	63	27	5	30	78	50	17	41	4	53
King C	50	26	7	32	90	35	54	58	6	46
King D	37	17	4	57	36	43	31	50	3	59
King E	30	16	5	20	39	28	11	15	4	49
King F	57	24	5	30	78	38	50	99	3	66
Mean		0.87		0.84		0.88		0.72		0.54
1.5	16	40	2	25	57	40	11	35	2	51
5033	17	08	3	10	20	33	20	45	2	58
6192	20	10	2	15	25	45	31	50	1	77
6885	20	10	2	20	29	51	31	50	2	51
1.96	17	08	2	20	39	33	20	45	2	51
Mean		0.60		0.71		0.90		0.71		0.41

TABLE 1b
Determination of Sensitivity of 11 Flavobacterium meningosepticum Strains

	Pyrolidino methyletracycline (Reverin®)		Ampicillin		Chloramphenicol		Streptomycin		Sulphadiazine	
	IC 50 µg/ml	log IC 50	IC 50 µg/ml	log IC 50	IC 50 µg/ml	log IC 50	IC 50 µg/ml	log IC 50	IC 50 µg/ml	log IC 50
King A	107	8.9	77	6.3	54	5.8	268	8.1	6061	12.6
King B	51	7.1	95	6.6	51	5.7	379	8.6	5654	11.5
King C	54	7.2	67	6.1	98	6.5	216	7.8	4280	12.1
King D	107	8.2	154	7.3	45	5.5	190	7.6	4371	12.2
King E	89	7.8	47	5.8	39	5.3	190	7.6	3021	11.6
King F	77	7.1	134	7.1	14	3.8	379	8.6	4766	12.1
sm	0.93		0.65		0.58		0.50		1.09	
L ₁	44	6.9	154	7.3	111	6.9	287	8.9	1516	10.6
Y933	61	7.4	95	6.6	77	6.3	287	8.2	2457	11.3
C692	61	7.4	209	6.8	63	6.0	144	7.2	1741	10.8
G58C	61	7.4	95	6.6	111	6.5	102	6.7	2143	11.1
T 396	61	7.4	154	7.3	73	6.0	574	9.9	1741	10.8
st	0.69		0.50		1.01		0.18		0.94	

Reverin® contains 37.5 per cent pyrolidinomethyl tetracycline. The values recorded have been corrected for this.

In order to evaluate the error involved in the method used the following statistical analysis was carried out.

The strains 5733 669^a 6886 and T 396 were considered to be identical as they had been isolated during one and the same hospital epidemic and were biochemically identical. L 5 was found to be more resistant to erythromycin and therefore excluded from the following calculations.

In the four identical strains the standard deviation s_b of log IC 50 was calculated for each antibiotic. These values are recorded nethermost in Table 1. The variances were tested according to Bartlett's test (see for instance Hall 1948) and did not differ significantly providing that the streptomycin value was excluded ($\chi = 4.90$ $f = 8$ $P > 0.35$). The greater variance noted in the case of streptomycin may be due to an experimental error. A mean standard deviation $s_{bf} = 0.74$ applying to the above mentioned four identical strains was thus obtainable if the streptomycin variance was excluded.

Correspondingly as regards the six meningitis strains a mean standard deviation $s_{mf} = 0.87$ was estimated. First the standard deviation of log IC 50 was calculated for each antibiotic as above. These values are recorded in the middle line in Table 1. Bartlett's test showed that values obtained for the six different strains as well as values for the different antibiotics did not differ significantly ($\chi = 11.72$ $f = 8$ $P > 0.05$).

Only the streptomycin variance differed significantly in the test

$$F = \frac{s_m^2}{s_b^2}$$

($F = 19.0$ $f_1 = 4$ $f_2 = 16$ $P < 0.0005$) and for the remaining antibiotics a mean standard deviation $s_{bm} = 0.81$ was calculated.

Combination of Two Antibiotics

The following tests were carried out on *kin*₅₀s serological type C strain the one most frequently isolated from premature children with meningitis.

Tests according to method 2a. Fusidin, pyrrolidinomethyl tetracycline, ampicillin, chloramphenicol, streptomycin and sulphadimidine were combined in all possible combinations using 20 μ g/ml of each antibiotic as described under method 2a. The culture became sterile only after combination of fusidin/chloramphenicol. This combination was further studied using the method described under 2c.

Tests according to method 2b. Table 2 shows the results after one and two days obtained by this method. As an expression of antagonism an increase in the titre of one log unit was found after two days using the combinations vancomycin/ampicillin and novobiocin/sulphadimidine. In streptomycin/erythromycin a titre increase of $\frac{1}{2}$ log unit was found. A fall in the titre was seen in a number of combinations marked in the case of erythromycin/fusidin. Combinations involving a fall in the titres after two days were also studied in accordance with the principles of method 2c.

Tests according to method 2c. Figs 1 and 2 illustrate results of the combinations erythromycin/pyrrolidinomethyl tetracycline and vancomycin/ampicillin. Fig. 1 depicts a potentiating effect. Fig. 2 an antagonizing effect.

TABLE 2

Results Obtained by Method *b* Combination of Two Antibiotics against *Flavobacterium meningosepticum* (King's Serological Type 1)

	day	log IC ₅₀	log 10 after addition of 20 µg/ml of the following antibiotics					
			bacitracin	chlor amphenicol	ampicillin	Pyrodoxine methyl tetracycline	Streptomycin	Sulpha dimidine
Prithromycin	1	2.5	0.5	1.5	2.5	1.5	2.5	2.5
	2	3.5	1.5	3.5	3.5	2.5	4.0	3.5
Neosoloxonin	1	3.5	0.5	2.5	2.5	1.5	3.0	3.0
	2	3.5	3.5	3.5	3.5	3.5	3.5	4.5
Penicillin	1	4.5	2.5	4.5	4.5	4.5	3.5	4.5
	2	4.5	3.5	4.5	4.5	4.5	4.5	4.5
Vancomycin	1	5.5	3.5	4.5	5.5	5.5	5.5	5.5
	2	5.5	4.5	5.5	6.5	5.5	5.5	5.5

Values in the column 1, the left represent log IC 50 of the four antibiotics stated in the column. Values recorded along the horizontal line represent log IC 50 after addition of 20 µg/ml of the six antibiotics recorded along the uppermost line.

TABLE 3
Results Obtained by a Combination of Two Antibiograms Using Method 2c

	Zone of Inhibition	Penicillin	Chloramphenicol	Novobiocin	Pyroglutamic acid methylester
Erythromycin	S (1/2+1/4)	S (1/8+1/4)	S (1/2+1/4)	S (1/2+1/8)	S (1/8+1/8)
Penicillin	A (1+1/16)	S (1/8+1/8)	0	S (1/4+1/4)	0
Chloramphenicol	S (1/2+1/4)	S (1/8+1/4)	S (1/2+1/4)	S (1/4+1/4)	0
Novobiocin	S (1/8+1/4)	S (1/8+1/4)	0	S (1/2+1/8)	0
Ampicillin	A (1/8+1/4)	S (1/8+1/4)	S (1/2+1/4)	S (1/4+1/4)	0
Streptomycin	S (1/4+1/4)	S (1/8+1/4)	S (1/2+1/4)	S (1/4+1/4)	0

Zone of Inhibition: A antagonism; 0 indifference. Fractions in brackets denote the fraction of the lowest inhibitory concentration of the two antibiotics found in the tube without growth in which the sum of the fractions is smallest.

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IDENTIFICATION OF *NEISSERIA GONORRHOEAE* BY MEANS OF FLUORESCENT ANTIBODY TECHNIQUE

By

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The use of Coons immunofluorescence technique (3-4) in the identification of *Neisseria gonorrhoeae* was first reported by Deacon *et al* 1959 (11). Subsequently further investigations in this field have been performed (6, 7, 12, 15, 20, 31, 34) but so far the fluorescent antibody technique has not been commonly used in the diagnosis of gonorrhoea. This technique and its possibilities as a routine method have been the subject of the following experiments, the preliminary results of which have been published previously (26).

MATERIALS AND METHODS

Preparation of Rabbit Anti Gonococcal Serum

Selection of rabbits. Young rabbits (1700-1900 g) with a negative gonococcal complement fixation test (25, 36) were selected for immunization. Samples of blood were collected and treated in the same manner as the corresponding samples of antisera.

Preparation of antigen. The antigen for immunization was made either from a single strain or from a pool of 20-30 strains of gonococci. Both fresh strains and well known stock strains were used. The strains were grown for 16-18 hours at 36°C in a moist atmosphere containing about 10 per cent CO₂. The medium was the fermentation medium described by A. Reyn (39) except that sugar phenol red and co-carboxylase were omitted. In some cases 2 units of Mycostatin[®] per ml were added to inhibit moulds. The bacteria were harvested in phosphate buffered saline (PBS) pH 7.2 (one litre made from 75 ml of 0.15 mol KH₂PO₄, 175 ml 0.15 mol NaHPO₄ and 750 ml 0.9 per cent NaCl) containing 3 per cent formalin (i.e. 3 ml solution formaldehyd plus 97 ml PBS). They were left for 30 minutes at room temperature, washed three times in PBS pH 7.2 and then resuspended in 0.3 per cent formalin in PBS pH 7.2 to a density corresponding to 2×10^8 bacteria/ml.

Immunization Schedules

A Groups of four to eight rabbits were immunized by intravenous injection of a dose increasing from 0.2 ml to 1.00 ml twice a week for four weeks. Five to six days after the last injection blood samples were collected by heart puncture. After a pause

Strains of *Staphylococcus aureus* were kindly supplied by Kirsten Rosendal MD, Department of Diagnostic Bacteriology, strains of *Escherichia coli* by Ida & Fritz Orskov MD, International Escherichia Centre, and strain of *Streptococcus pyogenes* by Beate Perhede PhD, Streptococcal Department (all Statens Serum Institut). For the clinical specimens I wish to thank A. Perdrup MD, Rudolph Berghs Hospital, Copenhagen, and Henning Schmitt MD, Rigshospitalet, Copenhagen.

containing 10 per cent CO₂. Next morning duplicate smears were made in a drop of tap water on carefully cleaned slides and the microscopic fields were marked with a glass diamond. Colonies suspected to be gonococci and/or several spots in confluent growth were touched with a platinum needle and transferred to the slides to form an appropriately thin smear. When no growth was visible the surface of the medium was scraped gently with the needle to catch any growth not yet visible to the naked eye (the lag phase may be prolonged for organisms which have been transported for some time). The smears were allowed to dry in the air, they were fixed for 5 minutes in methanol, dried and rehydrated in PBS pH 7.2 for 10 seconds.

Performance of the Test

The smears were placed in a moist chamber and the field covered with 1-2 drops of a mixture of conjugate and a pool of unlabelled normal rabbit sera or rabbit anti staphylococcal sera, the optimal ratio having been determined in preliminary experiments. Final dilutions of 1/8 of the conjugate and of 1/4 of the unlabelled serum were often found suitable. This modification known as the "one step inhibition test" (16-35) eliminated most of the non specific background staining and also the reaction with certain other microorganisms, especially certain staphylococcal strains which may be a source of error. The conjugate was diluted in saline when used without mixing with unlabelled serum.

The smears were incubated for 30 minutes at room temperature, rinsed thoroughly with PBS pH 7.2 using a washing bottle and placed for 10 minutes in PBS pH 7.2. The slides were dried with filter paper outside the field and mounted with phosphate buffered glycerol (9 parts of glycerol plus 1 part of PBS pH 8.2). Direct smears from secretions were treated in the same way except that they were mounted with a large cover glass to increase the field for microscopical examination.

If not read on the same day the preparations could be kept at 4°C for 3-4 days without any significant alteration in the degree of fluorescence taking place.

Microscopy

The microscopes were a Zeiss Standard and a Zeiss Universal microscope fitted with an ultra darkfield condenser and oil immersion objectives (apochrom 40 m I" and apochrom 100 m I). The light source was an Osram HBO 200 high pressure mercury lamp, the exciter filters were BC 38 and BG 12 (3 mm) and the barrier filter was No 47 or No 50 depending upon whether or not it was desirable to distinguish the blue autofluorescence exerted by several microorganisms. When an ultra darkfield condenser is used only slides with a thickness of 1 mm or less can be applied.

The degree of fluorescence was recorded in values from 0 to +++++. The values +++ and ++++ corresponded to the brilliant yellow green fluorescence which was observed in a specific staining reaction. The specific positive reaction was characterized by the morphology of the microorganisms in cases typical diplococci and the degree of fluorescence (++++ and +++++).

R E S U L T S

A Evaluation of the Specificity of the Fluorescent Antibody Test

1 Variations in the Specific Staining Reaction

The picture observed in the fluorescence microscope when a pure culture of gonococci was stained with FITC labelled antigonococcal globulin was found to vary in the following aspects:

a) *Age of culture* Gradually as a culture grew older the accurate picture of brilliantly fluorescent diplococci became blurred, the degree of fluorescence diminished and many abnormal forms were seen together with diffuse weakly stained masses. After 19 hours growth cul-

tures produced suitable preparations after 72 hours lysis was nearly complete. When primary cultures of material from patients were examined 24 hours growth was suitable presumably on account of a longer lag phase and in some cases perhaps also on account of some inhibitory effect of substances in the secretion from the patient (*eg* antibiotics).

Deacon *et al* suggested that subcultures would not be usable in the FAT since the reaction was thought to be due to a very labile surface antigen which was lost on subculturing (11). In order to investigate this suggestion three freshly isolated strains were selected which showed brilliant fluorescence of a thick even layer on each cell in the smear. These strains were subcultured daily. From each subculture smears were prepared for the FAT. After 16 transfers no alterations in the morphological picture nor in the degree or localization of the fluorescence were observed.

b) 'Serotypes' of the strains. Antisera against single strains or pools of strains produced after both schedule A and schedule B gave good conjugates in all cases. However each individual conjugate showed small variations in reactivity when tested against a large number of strains. No conjugate could be diluted further than 1:10 if a +++ or ++++ reaction was required also in relatively thick smears of gonococci. Single strains with their homologous conjugates gave a ++++ reaction in higher dilutions (1:40-1:80). These values were read at a magnification of 800 and a FITC protein ratio of 1:40 during the labelling procedure.

c) Batch of FITC. Different products of the dye produced conjugates which exerted varying degrees of fluorescence. Variation in the time and conditions of storage of the dye before use might also be of significance (21).

d) Antibiotic treatment of the patients and use of antibiotics in the medium. It is known that subinhibitory concentrations of antibiotics may induce morphological changes in gonococci (30). Most of such forms were well stained in the FAT. When they appear together with diplococci of normal appearance they do not cause diagnostic difficulties. On the other hand such forms are not likely to be recognized as gonococci in direct smears. Such strains in which morphological changes are brought about by the antibiotics in the medium are very rare.

2 Staining of *Neisseria (N)* other than *N. gonorrhoeae*

The strains shown in Table I were examined with two labelled rabbit antigonococcal sera. I) A pool of antisera against a stock strain of *N. gonorrhoeae* assumed to possess a comparatively high content of protein antigens (37-42). II) A pool of antisera prepared against a pool of 20 freshly isolated gonococcal strains.

TABLE 1

Stainability with FITC Labelled Antiserum to *N. gonorrhoeae*
Species within the genus *Neisseria*.

Strains	Stained with FITC labelled antiserum to		
	<i>N. gonorrhoeae</i> SS 1997/40 I	Pool of 20 strains of <i>N. gonorrhoeae</i> II	II mixed with unlabelled normal rabbit serum
<i>N. meningitidis</i> group A ATCC 13077	N	+ → + + +	N
<i>N. meningitidis</i> group B ATCC 13090	N	N	N
<i>N. meningitidis</i> group B ATCC 13091	N	N	N
<i>N. meningitidis</i> group C ATCC 13107	N	N	+ + → + + +
<i>N. meningitidis</i> group B ATCC 13113	N	+ + +	+ + +
<i>N. meningitidis</i> 11 strains isolated at SS	+ → + + + +	0 → + + + +	0 → + + + +
<i>N. catarrhalis</i> ATCC 8193	N	+ + +	N
<i>N. catarrhalis</i> ATCC 8176	N	+ + +	N
<i>N. catarrhalis</i> ATCC 7900	N	-	-
<i>N. catarrhalis</i> NCTC 4103	N	-	-
<i>N. catarrhalis</i> SS 465/2	N	N	N
<i>N. catarrhalis</i> SS 87895	N	+ + +	N
<i>N. flava</i> ATCC 14721	+ + +	+ + +	N
<i>N. perflava</i> ATCC 10555	N	N	N
<i>N. subflava</i> ATCC 11076	N	+ + +	N
<i>N. flavescens</i> ATCC 13120	-	N	N
<i>N. sicca</i> ATCC 9913	-	N	N
<i>N. haemolytica</i> ATCC 10379	N	N	N
<i>N. cuniculi</i> ATCC 14688	N	N	N
<i>N. cuniculi</i> var. <i>gigant</i> ATCC 14689	N	N	N
<i>N. canis</i> ATCC 14697	N	N	N
<i>N. caviae</i> ATCC 14659	N	N	N
<i>N. dentrificans</i> ATCC 14696	N	N	N
<i>N. species</i> SS 78438	N	-	-
<i>N. gonorrhoeae</i> (URI) ATCC 11688	-	N	N
<i>N. gonorrhoeae</i> (J G) ATCC 11689	-	N	N
<i>N. gonorrhoeae</i> (fresh control)	+ + + +	+ + + +	+ + + +

- + + + + Brilliant capsule like fluorescence typical appearance of specific staining
 + + + Ditto but less brilliant
 0 → + + + Variation in degree of fluorescence of bacteria in the same pure culture
 N Negative i.e. no or weak fluorescence
 - Not examined

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Furthermore the strains were tested in a one step inhibition test where conjugate II was mixed with the corresponding unlabelled serum from pre immune animals (normal rabbit serum)

As was to be expected the first species examined *N. meningitidis* showed a high degree of cross reactivity. This was most pronounced with the polyvalent antiserum and was not inhibited by normal rabbit serum

The second species *N. catarrhalis* which is also serologically related

to *N. gonorrhoeae*, did not cross react with conjugate I but some of the strains did so with the polyvalent conjugate II. This reaction was inhibited more or less by normal rabbit serum but in all cases to such a degree that the picture did not resemble a specific reaction.

In the third series which consisted of serologically less related strains only *N. flava* and *N. subflava* showed some cross reactivity in both cases the staining was inhibited by the addition of unlabelled normal rabbit serum.

The cross reaction with *V. meningitidis* was the only which was not inhibited by normal rabbit serum. The staining of meningococci was therefore tested both after absorption of the anti-gonococcal serum prior to labelling, and after absorption of the antigenococcal conjugate. For main killed suspensions of *N. meningitidis* Group A and a pool of *N. meningitidis* Group A B C and D were used for the absorption. By these procedures it was possible to prevent the staining of meningococci but at the same time the staining of gonococci was markedly reduced and the variability in the degree of fluorescence of different gonococcal strains was increased. Absorption with various tissue powders caused a decrease in the staining intensity of both gonococci and meningococci.

TABLE 2

Stainability with FITC Labelled Antiserum to *N. gonorrhoeae*
Species Considered as a Source of Error in Gram Stained Smears

Strains		Stained with FITC labelled antiserum to		
		<i>N. gonorrhoeae</i> SS 1997/40 I	Pool of 20 strains of <i>N. gonorrhoeae</i> II	II mixed with unlabelled normal rabbit serum
<i>Vibrio polymorpha</i>	ATCC 9907	N	N	N
<i>Vibrio polymorpha</i>	ATCC 10973	N	N	N
<i>Herellea species</i>	ATCC 9905	N	N	N
<i>Herellea species</i>	ATCC 9906	N	N	N
<i>Herellea species</i>	ATCC 11909	N	N	N
<i>Moraxella bovis</i>	ATCC 10900	N	N	N
<i>N. gonorrhoeae</i> (fresh control)		++++	++++	++++

++++ Brilliant capsule like fluorescence typical appearance of specific staining
N Negative i.e. no or weak fluorescence

3 Staining of Species not Belonging to the Genus *Neisseria*

Table 2 shows a series of microorganisms which in Gram stained smears often have been considered as sources of error in the diagnosis of gonorrhoea. It will be seen from the Table that these bacteria did not cause any difficulty in the FAT.

Table 3 shows the results of examination of microorganisms the majority of which may occur in cultures from specimens to be examined

for gonococci. The main problem was caused by certain staphylococcal strains because of the similarity in morphology and the intensity of staining. The reaction was eliminated by the admixture of normal rabbit serum. Furthermore, such strains never occurred in specimens received for identification of gonococci except the one which revealed the problem and which originated from a male urethra. One hundred and one strains of *Staphylococcus albus* were tested. These were isolated from cultures intended for the delayed FAT. Since no *Staphylococcus aureus* were found in these cultures, all the strains listed were kindly provided by Dr Kirsten Rosendal, Department of Diagnostic Bacteriology, Statens Seruminstitut.

Occasionally other microorganisms e.g. yeasts were stained but these were easy to exclude because of their differing morphology.

TABLE 3
Stainability with FITC labelled Antiserum to *N. gonorrhoeae*
Various Unrelated Species

	Number of strains examined	Stained with FITC labelled		
		Normal rabbit serum	Anti gonococcal serum	Anti gonococcal serum mixed with unlabelled normal rabbit serum
<i>Staphylococcus albus</i>	101	-	✓	-
<i>Staphylococcus aureus</i>	115	-	✓	-
<i>Staphylococcus aureus</i>	163	-	-	-
<i>Staphylococcus aureus</i>	49	++++	++++	✓
<i>Streptococcus pyogenes</i> group A	4	+ → +++++	+ → +++++	✓
<i>Streptococcus pyogenes</i> group B	5	✓	✓	✓
<i>Streptococcus pyogenes</i> group C	4	+ → +++++	+ → +++++	✓
<i>Streptococcus pyogenes</i> group D		+ → +++++	+ → +++++	✓
<i>Escherichia coli</i> type O1-O25	2	✓	✓	-
<i>Proteus mirabilis</i>	6	-	✓	-
<i>Aerobacter cloacae</i>		-	✓	-
<i>Haemophilus vaginalis</i> (1 page)	1	-	✓	✓
<i>Pasteurella multocida</i> ATCC 10244	1	-	✓	✓
<i>Pasteurella</i> strains isolated from rabbits ()	4	-	✓	✓
<i>N. gonorrhoeae</i> (fresh control)		✓	++++	++++

Except one strain which showed a +++++ reaction the experiment has not yet been repeated.

++++ Brilliant capsule like fluorescence typical appearance of specific staining
+ → +++++ Variations in degree of fluorescence of bacteria the same pure culture

Negative i.e. no or very faint fluorescence

Not examined

By Dr A. Regn. reference No. 36

B Application of the FAT in the Diagnosis of Gonorrhoea

Preliminary experiments were performed on smears of urethral discharge from male patients suspected to be suffering from gonorrhoea. On account of a brilliant non specific staining of leucocytes and occasional staining of microorganisms other than gonococci the identification of gonococci was found to be very difficult. A satisfactory reduction in the non specific staining of leucocytes was not achieved by absorption of the conjugates with various tissue powders human erythrocytes or human leucocytes in addition the specific staining of gonococci was more or less reduced. The application of different counterstaining techniques was also examined. The effect of Issamine rhodamine RB 200 labelled albumin (40) was found to be variable and insufficient. Normal globulins labelled with either Issamine rhodamine RB 200 or TMRITC (tetramethylrhodamine isothiocyanate (BBL)) gave a more even red background but neither of the two conjugates reduced satisfactorily the non specific staining of leucocytes. The same poor results were obtained with Evans blue (14) and Flazo Orange (18-19). With all the methods the counterstain occasionally interfered with the specific staining of gonococci giving them all shades of colour from red to yellowish green.

Ovcinnikov (33) suggested that digestion of the smears with trypsin prior to staining would solve the problem. In our hands trypsin treatment diminished not only the non specific staining of leucocytes but also reduced considerably the specific staining of gonococci.

The effect of alteration of the ratio between dye and protein during the labelling procedure in combination with chromatography on DEAF cellulose will be described in a subsequent paper. These experiments and those carried out by other authors showed that gammaglobulins purified on DEAF cellulose after labelling would never give a non specific staining of leucocytes and that this staining was caused by over labelled globulin molecules (5-13-27). The latter point explains why it was not possible to block the staining of leucocytes by the addition of unlabelled normal rabbit serum to the conjugate.

Nevertheless the diagnosis made on direct smears from discharge still remained problematic since the intracellular gonococci were never stained and the extracellular gonococci often occurred sparsely being only stained brilliantly when not covered by secretion.

The following experiments with specimens from patients were performed using a modification of Deacon's technique (12). Duplicate specimens were kindly supplied by the physicians at the Venereological Out Patients Department of Rudolph Berghs Hospital Copenhagen and by Dr Hennings Schmidt Department of Dermatology and Venereology Rigshospitalet Copenhagen. The transportation time never exceeded 4-6 hours. One swab was used for routine culture the diagnosis being confirmed by Gram staining, fermentation reactions and oxidase

reaction. This routine result was available after 2-3 days at the earliest. The other swab was used for preparation of two smears for undelayed FAT and for inoculation on the routine medium followed by preparation of smears for delayed FAT as described under Materials and methods.

The duplicate specimens were received during two periods. The results from the first period have been published previously and a few cases described in detail (26). A total of 664 duplicate specimens were examined corresponding to 170 female and 161 male patients. Among 664 specimens 178 were found positive by culture and 205 positive by the FAT. This corresponds to an increase of 15 per cent in positive findings by the FAT. 66 male patients were found positive by both methods. 65 female patients were found positive by culture against 67 by the FAT. In the case of the males only specimens from the urethra were received. Three specimens were taken from each female patient viz. from the urethra, the cervix and the rectum. The results are shown in Table 4. If the FAT were used the increase in positive specimens from the three sites in the female material was about 24 per cent—major part of this yield being from the more heavily contaminated rectal specimens.

TABLE 4
*Identification of *Neisseria gonorrhoeae* in 170 Female Patients
Comparison of FAT and Culture*

	Patients		Urethra		Cervix		Rectum		All sites	
	FAT	culture	FAT	culture	FAT	culture	FAT	culture	FAT	culture
Pos. FAT & pos culture	64	64	44	44	56	56	7	7	107	107
Pos FAT & neg culture	3		9		8		17		37	
Neg FAT & pos culture		1		3		2		0		3
Total positive sites			53	47	64	58	24	7	139	117
Total positive patients	68		54		64		24			

During the last part of the second series the hospitals supplied an extra smear corresponding to each specimen. These smears were used for Gram staining and compared with the results of the undelayed FAT. By these methods a total of 48 specimens and smears from 18 male patients and 171 specimens and smears from 58 female patients were examined.

Among the 48 male patients the same 30 patients were found positive both by culture and by delayed FAT. Out of these 30 patients 28 were found positive by the undelayed FAT, one patient was negative and from another both slides were lost. The corresponding Gram stained smears showed 19 positive and contained extracellular Gram neg.

II Application of the FAT in the Diagnosis of Gonorrhoea

Preliminary experiments were performed on smears of urethral discharge from male patients suspected to be suffering from gonorrhoea. On account of a brilliant non specific staining of leucocytes and occasional staining of microorganisms other than gonococci the identification of gonococci was found to be very difficult. A satisfactory reduction in the non specific staining of leucocytes was not achieved by absorption of the conjugates with various tissue powders human erythrocytes or human leucocytes. In addition the specific staining of gonococci was more or less reduced. The application of different counterstaining techniques was also examined. The effect of Lissamine rhodamine RB 200 labelled albumin (40) was found to be variable and insufficient. Normal globulins labelled with either Lissamine rhodamine RB 200 or TVRITC (tetramethylrhodamine isothiocyanate (BBI)) gave a more even red background but neither of the two conjugates reduced satisfactorily the non specific staining of leucocytes. The same poor results were obtained with Evans blue (14) and Flazo Orange (18-19). With all the methods the counterstain occasionally interfered with the specific staining of gonococci giving them all shades of colour from red to yellowish green.

Oucinnikov (38) suggested that digestion of the smears with trypsin prior to staining would solve the problem. In our hands trypsin treatment diminished not only the non specific staining of leucocytes but also reduced considerably the specific staining of gonococci.

The effect of alteration of the ratio between dye and protein during the labelling procedure in combination with chromatography on DEAE cellulose will be described in a subsequent paper. These experiments and those carried out by other authors showed that gammaglobulins purified on DEAE cellulose after labelling would never give a non specific staining of leucocytes and that this staining was caused by over labelled globulin molecules (5-13-27). The latter point explains why it was not possible to block the staining of leucocytes by the addition of unlabelled normal rabbit serum to the conjugate.

Nevertheless the diagnosis made on direct smears from discharge still remained problematic since the intracellular gonococci were never stained and the extracellular gonococci often occurred sparsely being only stained brilliantly when not covered by secretion.

The following experiments with specimens from patients were performed using a modification of Deacon's technique (12). Duplicate specimens were kindly supplied by the physicians at the Venereological Out Patients Department of Rudolph Berghs Hospital Copenhagen and by Dr Henning Schmidt Department of Dermatology and Venereology Rigshospitalet Copenhagen. The transportation time never exceeded 4-6 hours. One swab was used for routine culture the diagnosis being confirmed by Gram staining fermentation reactions and oxidase

The above mentioned experiments were performed on a material which in some respects must be considered as selected viz 1) The transportation time was short namely 4-6 hours and 2) the specimens were collected by experienced venereologists from a group of patients with a high incidence of gonorrhoea. Normally specimens are also transported in modified Stuart medium and the transportation time very seldom exceeds 24 hours. The culture method used for routine diagnosis reveals about 10 per cent positives out of 110 000 specimens per year. In order to get an impression of the practicability of the FAT in the routine work 821 of the swabs received were inoculated on two plates one for the usual procedure and one for the delayed FAT. The FAT was given a handicap as the medium for routine culture was always inoculated first. The FAT was performed on a single smear from each culture for 502 specimens and on duplicate smears for the following 319 specimens.

The 502 single smears corresponded to 91 male patients and 184 female patients. Among the 91 male patients 12 were found positive by both methods and 2 by the FAT alone. The 184 female patients showed 23 positive results by both methods 2 positive by the FAT alone and another 3 positive by culture alone. The number of specimens from the 184 female patients was 409 with 31 positive results by both methods 7 positive by the FAT alone and 6 positive by culture alone.

The 319 duplicate smears were from female patients only. 149 specimens were found positive by culture and 163 by the FAT i.e. an increase of about 9 per cent.

DISCUSSION

During recent times the most important applications of immunofluorescence in diagnostic bacteriology have been reviewed by Cherry & Woody (1). They point out that immunofluorescence of bacteria has only two dimensions—morphology and serological specificity and further lack of specificity can be tolerated only if the morphology of the cross reacting antigen clearly distinguishes it from the specific antigen. In the present immunofluorescent studies of gonococci it was not found possible to fulfil these conditions. The known serological cross reactivity within the genus *Neisseria* was reflected in the stainability of species other than *N. gonorrhoeae* with FITC-labelled gonococcal antisera resulting in morphologically identical pictures. It was possible to inhibit the weaker reactions by the addition of normal rabbit serum. However the stronger reactions observed in some meningococcal strains could not be eliminated in this way. Absorption of the conjugates with meningococci was effective but at the same time the degree of staining of the gonococci was diminished presumably due to the competition of a high content of soluble meningococcal antigens in the absorbed conjugates. Such antigens were easily demonstrable by

the Ouchterlony gel precipitation technique (unpublished data). From a practical point of view the problem is not serious since meningococci are seldom found in specimens received for identification of gonococci. A greater problem was the strong reactivity shown by about 10 per cent of all the strains of *Staphylococcus aureus* examined organisms in which the morphological differences from *N. gonorrhoeae* are too delicate to be differentiated in immunofluorescent stained smears. This problem has occurred to nearly all workers using FAT for bacteriological diagnosis independent of the antigen antibody system involved (1, 2, 6, 21, 27, 15). Danielsson (9) used absorption of the anti gonococcal conjugate with a strongly cross reacting strain of *Staphylococcus aureus*. It is logical to do so if the reaction can be considered as an expression of antigenic relationship or as a detection of naturally occurring antibodies. According to the author's experience with this method of absorption the reduction in staining the gonococci was of a similar degree or even more pronounced than the reduction in staining the staphylococci. This might be expected if the staining is considered not as a result of an antigen antibody reaction but as a result of a non specific adsorption of globulins to the surface of certain staphylococci (27, 28). This consideration is also in agreement with the fact that the addition of a surplus of non labeled normal rabbit serum to the conjugate has proved to be an easy and efficient way of blocking the staining of staphylococci. Thus although the morphology of the cross reacting antigen did not clearly distinguish it from the specific antigen it is possible to obtain a reliable result when working under known conditions.

At the beginning of the discussion Deacon, Peacock, Freeman & Harris (11) state the following in their first paper: "Our evidence appears to indicate that *N. gonorrhoeae* possesses a heretofore unrecognized antigenic component similar in character to the antigen of *S. typhi* or the antigen of the Fischerich group. The new antigen appears to be species specific and may be recognized by slide agglutination tests employing living or formalin killed cultures and by fluorescent antibody techniques. This new antigen which was assumed to be associated with specific staining in the FAT as well as with the virulence of the microorganism was also associated with freshly isolated nonagglutinable gonococcal cultures."

In the above mentioned paper the authors did not distinguish between various forms of nonagglutinability. The nonagglutinability was described in two connections: 1) as confirming the observations of Wilson (42) and 2) as revealing an antigen referred to as GC-k(B). Apparently this implies a contradiction since the data published comprise only the demonstration of O nonagglutinability of some recently isolated strains. Wilson describes nonagglutinability of a recently isolated strain in an antiserum against another living, recently isolated

strain an observation which probably reflects a type variation and not the presence of a species specific antigen which is easily lost on subculture. On the other hand an antigen of type $\text{h}(\text{B})$ is revealed by its O inagglutinability (41).

On account of the important properties said to be connected with inagglutinability attempts were made to isolate such inagglutinable strains. Antisera against both formalin killed and heated bacteria of two recently isolated and two stock strains of *A. gonorrhoeae* were available. For all these strains O inagglutinability was demonstrated; the antisera showed a similar degree of specificity irrespective of whether they were used in the FAT or tested in complement fixation test against antigens of different species of *Neisseria*. Using antisera against formalin killed gonococci in the agglutination test 93 per cent of 201 living cultures of *A. gonorrhoeae* were found to be autoagglutinable. This percentage was diminished although not encouragingly when formalin killed suspensions were used. Further examination of possible inagglutinability was not performed.

The finding that older gonococcal cultures are poorly stained in the FAT has been confirmed by all workers in this field. This does not appear to mean a loss of antigen (h antigen) since a new subculture 16-18 hours old exhibits the usual brilliant fluorescent picture as shown by smears from the primary culture. In 16 subsequent subcultures three strains selected at random were followed without any change in morphology or in staining intensity. Danielsson (8) has controlled the staining ability after up till 102 subcultures without being able to demonstrate any loss of antigenic components important for the staining reaction. In other experiments (8) he finds that heat labile as well as heat stable antigens take part in the reaction in the FAT. In agreement with the latter observation FITC labelled monovalent anti serum against reference strain VI (11413) has proved to be highly valuable for diagnostic purposes. This strain is known to contain several heat stable antigens of *Neisseria gonorrhoeae* (37).

That this new $\text{h}(\text{B})$ antigen should possess characteristics similar to VI antigen of *Salmonella typhi* has been questioned first by Otis and Koo (33) and later indirectly in publications from Deacon's laboratory (22-23). In this laboratory the virulence of gonococci was attributed to the type of colony. No agreement was found between colony type and any serological reaction nor between colony type and stainability in the FAT. All four colony types were specifically stained by FITC labelled antiserum against the virulent type I as well as the avirulent type IV colony. This excludes definitely the original conception that the staining reaction was linked to a labile virulence associated surface antigen.

For diagnostic purposes the ideal fluorescent antibody technique was originally thought to be the direct staining of specimens (smears) with

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BRIEF REPORT

PERIPHERAL NERVE CHANGES IN TAY SACHS AND BATTEN SPIELMEYER VOGT DISEASE

A preliminary report

By Kristin Kristensson, Ingie Olsson and Patrick Sourander

In Sweden two well defined types of familial amaurotic idiocy, i.e. the infantile or Tay Sachs disease (TSD) and the juvenile or Batten Spielmeier Vogt disease (BSVD) have repeatedly been reported (cf Feg Olofsson *et al* 1966 Sjogren 1933 Rayner 1962). Recent chemical (Sorennerholm 1963), histochemical (Zeman & Albert 1963 Kristensson & Sourander 1967) and ultrastructural (Zeman & Donahue 1963) investigations have shown that in spite of certain common clinical and light microscopical features these two conditions must be regarded as separate nosological entities. Although the changes in the central nervous system are well known no information exists about the state of the peripheral nerves in these diseases.

In the past the study of peripheral nerves in neurolipidoses has been much neglected. However it has recently been shown that both in metachromatic (MLD) and globoid cell (GCD) leucodystrophy the peripheral nerves are severely involved, probably as a result of a basic metabolic disturbance affecting the whole nervous system (Sourander & Olsson 1967 Allen & de Veyra 1967).

Considering the frequent use of peripheral nerve biopsies for the diagnosis of MLD and the recently detected peripheral neuropathy in GCD a histological scrutiny of peripheral nerves in other neurolipidoses including various types of amaurotic idiocy seems justified.

Materials and Methods. Autopsy material from one case of TSD and three cases of BSVD were examined. Patho-anatomical data concerning the central nervous system and the visceral organs in the TSD case including chemical findings have been presented elsewhere (Feg Olofsson *et al* 1966). However in this paper the peripheral nerve changes were overlooked. Corresponding information about BSVD is to be published (Kristensson Rayner Sourander Sorennerholm). Paraffin embedded specimens from the spinal cords and the proximal part of the sciatic nerve were stained with luxol fast blue cresyl violet and impregnated with silver according to Palmgren for axons. Spinal nerve roots, one dorsal root ganglion and the oculomotor nerve from the TSD and one dorsal root ganglion from one of the BSVD cases were treated in the same way.

Results

Tay Sachs disease. The motor neurons of the cord presented changes similar to those seen in the cerebral cortex (Feg Olofsson *et al* 1966), i.e. marked distension of the cytoplasm and of some processes indicating lipid storage. The white matter of the cord showed severe destruction of the axons and myelin sheaths in the ventral and lateral tracts whereas the dorsal tracts were better preserved. A moderate degeneration of myelin sheaths and of axons occurred both in the ventral and dorsal roots. Loss of neurons associated with proliferation of capsular and interstitial cells were frequently observed in the dorsal root ganglia. Remaining neurons showed variable swelling of the cytoplasm.

The sciatic nerve showed an almost total destruction of myelin sheaths associated

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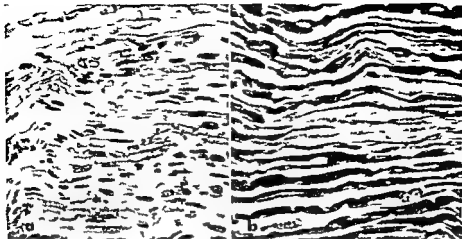


Fig 1

Sciatic nerve from the TSD case a) Luxol fast blue cresyl violet
b) Impregnation with silver for axons

with a crowding of Schwann cell nuclei (Fig 1a). One remarkable finding was the frequent occurrence of irregular elongated thickenings of the axons (Fig 1b). However, compared to the severe lesions of the myelin sheaths, the axonal changes were quite modest. The extracerebral part of the oculomotor nerve showed changes resembling those of the sciatic nerve.

Batten-Spielmeier-type disease. A marked neuronal storage of lipofuscin and some loss of neurons was present in the anterior horns. No obvious changes were seen in the white matter of the cord. Marked loss of neurons associated with proliferation of capsular and interstitial cells were present in the dorsal root ganglion. In the remaining neurons lipofuscin was stored.

A very large number of the myelin sheaths were destroyed in the sciatic nerves in all cases. In one of them the destruction was partly of segmental type. The axons in two of the cases showed extensive fragmentation, but in the remaining case the degeneration was only slight.

Discussion

In view of the chemical differences between TSD and BSVD it is appropriate to discuss separately the peripheral nerve change observed in these diseases.

In TSD a tenfold increase of the typical CGF ganglion side in the spinal roots has been detected by Jennerholm (Feg Olofsson et al 1966). Since not only the perikaryon of neurons but also the axons in the central nervous system show swellings due to gangliosidosis, the possibility exists that the thickened axons of the peripheral nerves and the increased content of ganglioside is not possible to decide whether there is a relation of the myelin sheaths secondary to a disturbed relation between the myelin and the axon or induced by altered metabolism of the Schwann cells.

A very plausible explanation for the extensive degeneration of nerve fibres in BSVD can be considered. The thinning of axons and myelin in the peripheral nerves in two of the cases may be due to the degeneration of neurons in the anterior horns of the spinal cord. It is difficult to distinguish between the peripheral and central factors during the development of the disease. Still the possibility remains that the peripheral nerves are connected with a local metabolic disturbance. The relation at the pathogenesis of the peripheral nerve lesions in TSD and BSVD must be investigated by conventional methods, but call for further investigation including histochemistry and electron microscopy applied to biopsy material from peripheral nerves in TSD and BSVD.

Summary

Previously unrecognized peripheral nerve changes in one case of Tay Sachs disease and in three cases of Batten-Spielmeyer-Vogt disease are described and discussed

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BRIEF REPORT

ISOLATION OF ADENOVIRUS FROM A SKIN VESICLE AND CEREBROSPINAL FLUID FROM A PATIENT SUFFERING FROM STEVENS JOHNSON SYNDROME

By Pirlla Pohjanpelto and O Pettay

Several factors including administration of long acting sulphur preparations and infection with herpes simplex or mycoplasma have been connected with Stevens Johnson syndrome (1). Demonstration of rise in complement fixing antibodies against adeno group antigen in two patients suffering from Stevens Johnson syndrome suggest that also adenoviruses may in some case play a role in manifestation of this syndrome (2). In this paper a report is given of isolation of adenovirus type 3 from a skin vesicle and cerebrospinal fluid from a patient hospitalized on account of Stevens Johnson syndrome.

On the suspicion of urinary infection the patient a girl of five years received nitrofurantoin and sulphur metoxipridazine nine days after the first administration of sulphur preparations the patient developed a high fever 39°C and a few days later vesicles appeared both in the mucous membranes and the skin. The patient was somnolent and sometimes unorientated. The cerebrospinal fluid was clear it contained 15 mg protein 90 mg% glucose and one cell/μl.

From a skin vesicle and from the cerebrospinal fluid an agent was isolated that caused degeneration of V cells (continuous human amnion cell line) fixed complement in the presence of adenovirus immune serum and agglutinated red cells from a chimpanzee. Haemagglutination was inhibited by type 3 adeno immune serum. The titre of complement fixing adeno antibodies in the serum of the patient was 1:64. Any rise in the titre of the antibodies was not demonstrated in the course of the illness.

Isolation of adenovirus type 3 from a skin vesicle and from the cerebrospinal fluid in a patient suffering from Stevens Johnson syndrome is in support of the hypothesis that adenovirus in some cases may play a role in the aetiology of this syndrome.

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Immunology

Nilsson U R Institute of Medical Microbiology University of Lund Sweden and
Scripps Clinic and Research Foundation La Jolla Calif USA STUDIES ON
THE MODE OF ACTION OF THE FIFTH SIXTH AND SEVENTH COMPONENTS
OF HUMAN COMPLEMENT (C) IN IMMUNE HEMOLYSIS

The classical third component of C is presently considered to consist of at least six different factors C3 C5 C6 C7 C8 and C9 These studies concerned the mode of action of C5 C6 and C7 As a group these components were found to interact with sensitized sheep erythrocytes (E1) which have reacted with the first fourth second and third components of C EAC1a 4 2a 3 5 6 7 The reaction product was designated EAC1a 4 2a 3 5 6 7

Kinetic analysis indicated the reaction sequence C5-C6-C7 Isolation of the intermediate complexes corresponding interaction of EAC1a 4 2a 3 with C5 or with C5 and C6 at physiological ionic strength were unsuccessful Formation of EAC1a 4 2a 3 5 6 7 proceeded only when C8 C6 and C7 were present simultaneously in the reaction mixture This interdependence between the three components suggested fluid phase interaction Protein protein interaction was demonstrated between C5 and C6 and C5 and C7 by density gradient ultracentrifugation Dose response studies furnished support for the hypothesis that C5 C6 and C7 constitute a functional unit

This work was done in collaboration with Dr H J Muller Eberhard at Scripps Clinic and Research Foundation La Jolla Calif

Holm G & Johansson Bo the Wenner Gren Institute Stockholm IN VITRO STUDIES OF LYMPHOCYTES FROM PATIENTS WITH HODGKIN'S DISEASE AND CHRONIC LYMPHATIC LEUCEMIA

To be published in *Clinical Experimental Immunology* 1967

Magnusson Fernilla & Kallings L O The Department of Virology Karolinska Institutet and the Department of Bacteriology National Bacteriological Laboratory Stockholm ANTIBODY SPECTRUM OF HUMAN GAMMA GLOBULIN FOR CLINICAL USE

Human gammaglobulin is widely used for prevention and treatment of a vast variety of infectious conditions I estimate the all of this administration it was so ind imperative to have a better knowledge of the morphological spectrum of antibodies present and to what extent the different biological gammaglobulin are

similar in composition. In this study 10 batches of human gammaglobulin have been tested for the ability of neutralizing an assortment of infectious agents generally occurring in Sweden. It is also speculated on whether gammaglobulin representing a great number of blood donors could be used for a rough epidemiological screening. A more comprehensive report on this study will be presented elsewhere.

Jonsson Jonas & Fagraeus Astrid Department of Immunology, National Bacteriological Laboratory, Stockholm. THE MIXED HAEMADSORPTION TECHNIQUE AS A DIAGNOSTIC TEST FOR THYROID ANTIBODIES

If circulating thyroid antibodies have a pathogenic effect by damaging living thyroid cells they must in the first place be assumed to exert this effect by attacking the cell membranes. The mixed haemadsorption technique applied to monolayer cultures of thyroid epithelial cells (1) has proved to be a convenient method for the demonstration of such antibodies especially in large scale examinations. Sera from 259 cases of thyroid disease, 34 cases of connective tissue disease and 347 apparently healthy controls were examined with this test. A reaction in the serum dilution 1/1600 or more indicated a diagnosis of chronic thyroiditis or thyrotoxicosis with a probability of 80 per cent. The probability that a reaction at this titre level should occur in the absence of thyroid disease was 6.4 per cent. The mixed haemadsorption reactions obtained were of two types called ring zone and filled zone reactions. Ring zone reactions were obtained with 2/3 of the sera from cases of chronic thyroiditis, 1/3 of the sera from cases of thyrotoxicosis, less than 10 per cent of the sera from other thyroid diseases and with 3 per cent of the control sera. There was a strong correlation between the ring zone reaction and a reaction with cytoplasmic antigen in the immunofluorescent test. The organ specificity of the reaction was confirmed by tests with other types of human cell cultures and by absorption experiments. The incidence of thyroid antibodies in patients' sera as well as in the control sera varied with age and sex in the same way as in the common standard tests for thyroid auto-antibodies. The thyroid antibodies demonstrated by the mixed haemadsorption technique thus have the same diagnostic implications as those which are demonstrated by the immunofluorescent test and the complement fixation test. The results reported above suggest that the antigen-antibody system that gives rise to the ring zone reaction is more specific for chronic thyroiditis than others. Compared to the diagnostic tests employed so far the mixed haemadsorption test seems to increase the possibilities to differentiate serologically between chronic thyroiditis, thyrotoxicosis and atrophic colloid goiter.

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Lundbeck H & Tirunavaran M O National Bacteriological Laboratory, Stockholm. ANTIBODIES AGAINST LIPASE FROM STAPHYLOCOCCI

A method for determination of staphylococcal lipase with the aid of egg yolk agar plates has been worked out and published previously. The enzyme suspension to be tested is filled into a well cut out in the agar and allowed to diffuse into the agar at 37°C. Lipase activity is observed as a clarification and precipitation zone surrounding the well. The diameter of the zone can be used to quantitate the lipase activity. The reaction is inhibited by sera containing antibodies against lipase.

technique for determination of antilipase antibodies with the aid of standardized quantities of lipase has been worked out. This technique was used to demonstrate the occurrence of antilipase antibodies in human sera from different age groups and to study the correlation between antilipase and antistaphylolysin antibodies. It was found that the level of antibodies against lipase decreased with age from 0 to 6 months, indicating the presence of maternal antibodies, and increased with higher age reaching a constant level in groups above 10 years. There was a good correlation between antilipase and antistaphylolysin titers. The antilipase activity was localized in the 7S gammaglobulin fraction of serum.

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A RADIOIMMUNOLOGICAL METHOD FOR DETERMINATION OF PROTEINS WITH THE USE OF SEPHADEX COUPLED ANTIBODIES

A new modification of the radioimmunoassay for proteins was presented (J. Wile & J. Porath, *Biochim. Biophys. Acta* 130 (1966) 257-260). The immunoglobulins in an antiserum were precipitated by ammoniumsulphate and chemically coupled to an insoluble polymer, a Sephadex derivative (isothiocyanatophenoxymethylpropyl Sephadex) prepared according to Axén and Porath (*Experientia* 210 (1966) 387). The immunosorbent thus prepared may be freeze dried. The antigen binding capacity of the antibody Sephadex particles was not altered after 12 months storage at +4°C. One gram antibody Sephadex particles could be used for more than 10,000 reactions. In the radioimmunoassay the sample to be investigated, the antigen labelled with a radioactive isotope (^{125}I) and the antibody Sephadex were added to a test tube and incubated for 24 hours by slowly rotation around a horizontal axis. The antibody bound labelled antigen was separated from the unbound by centrifugation. The radioactivity in the Sephadex precipitate was counted in a scintillation detector. The sensitivity of the assay could be increased by reincubation of the antibody Sephadex with the solution to be investigated prior to the addition of the labelled hormone and incubation for several days. In this way less than 1 pg of luteinizing hormone per ml solution could be detected. The technique has hitherto been applied to the assay of insulin, growth hormone, chorionic gonadotrophin, luteinizing hormone and immunoglobulin C. The high sensitivity, precision and simplicity of this method make it as suitable for scientific purposes as for routine hospital laboratory use.

Brilman S. Institute of Tumor Biology, Karolinska Institutet, Stockholm. STUDIES ON THE PERSISTENCE OF IMMUNOGENICITY OF SOLUBLE AND PARTICULATE ANTIGENS IN THE MOUSE

The persistence of immunogenicity in the mouse of both red blood cell antigens and bacterial lipopolysaccharide antigens of *Escherichia coli* B5 was determined utilizing a transfer system. Mice were immunized with the respective antigen and after different time intervals the mice were lethally irradiated and repopulated with syngeneic normal lymphoid and bone marrow cells. The induction of a primary antibody response in the transferred cell population indicated the retained immunogenic material in the recipient. In this system it was shown that the immunogenicity of SRBC antigens persisted for 14 days whereas the bacterial antigen persisted for more than 45 days.

A protein antigen, human serum albumin (HSA), was studied in a modified transfer

fer system Utilizing the notion that soluble HSA does not induce a primary response in the mouse but triggers a secondary mice were injected with small amounts of soluble HSA and after different time intervals injected with preimmunized syngeneic spleen cells The induction of a secondary antibody response would indicate retained immunogenic HSA The half life of immunogenicity of HSA measured by this method and the half life of radioactively labelled HSA in serum of the normal mouse did not differ statistically from each other

Virology

Norby E & Wadell G Department of Virus Research Karolinska Institutet Stockholm SOLUBLE COMPONENTS OF ADENOVIRUS TYPE 4

To be published in *Virology* 1967

Stehag S F & Bloth B Department of Immunology National Bacteriological Laboratory and Department of Virus Research Karolinska Institutet Stockholm EXAMINATION OF VIRUS GAMMA M ANTIBODY COMPLEXES BY ELECTRON MICROSCOPY 1 CONFIGURATION OF GAMMA M ANTIBODIES AND MODE OF ATTACHMENT TO ANTIGEN

Poliovirus γ M antibody complexes were examined by the negative staining technique in the electron microscope The virus was purified by equilibrium centrifugation and rabbit γ M antibodies by zone centrifugation or gel filtration The specificity of the poliovirus γ M antibody reaction was established by the use of heterologous antigen and γ M fractions from normal sera γ and II virus particles aggregated separately in the presence of γ M antibodies against both these antigens

The size of specific poliovirus aggregates and the density of the cross linking network of γ M antibodies increased with increasing relative γ M antibody concentrations Employing intermediate or rather high relative antibody concentrations single virions or virions in the periphery of aggregates were often surrounded by an aura of loops The maximal length of the turn of the loops was 220 to 250 Å and their width 30 to 40 Å suggesting that they represented γ C like 7S subunits of the γ M molecule The maximal number of subunits contained in one γ M molecule appeared to be 5 or 6 The distribution of the lengths of γ M antibodies bridging virions had a mode and median of about 330 Å and maximal value of 360 to 370 Å

Biberfeld Gunnar & Fagraeus Astrid National Bacteriological Laboratory Stockholm FARLY SEROLOGIC DIAGNOSIS OF RABIES

Antibodies were demonstrated in rabiesinfected dogs during the incubation period Five dogs were inoculated intramuscularly with street virus (Dr Schmidt Atlanta) and blood specimens were collected every third day The dogs fell ill 10-17 days after inoculation and died 1-5 days later Antibodies against rabies were tested with fluorescent antibody (FA) and mixed haemadsorption (MH) technique in this laboratory and by serum neutralization (SN) by Dr Schmidt The antigen for the FA test consisted of green monkey kidney (CVN) cells infected with rabiesvirus (Flury HEP HDGS) and centrifuged directly on to slides in a special rotor After fixation in acetone the antigenpreparations could be stored at -20°C for at least two months FA antibodies (titres 1/10-1/40) were demonstrated in 4 of 5 dogs 4-10 days before onset of illness and in the fifth dog on the day of clinical onset Four

of the dogs also developed MH and SN antibodies No antibodies were found 1-3 days after inoculation

Using a fluorescein-conjugated antidog gamma globulin serum rabies antibodies were also demonstrated in a fox injected with two doses of live Flury HEP vaccin

In addition a rise in FA antibodies from $< 1/5$ to $1/80$ was demonstrated in a patient given a full treatment with sheep brain vaccin (Lister Institute)

Tirunaryanan V & Lundback H National Bacteriological Laboratory Stockholm
RELATIONSHIP OF pH TO SYNTHESIS OF STAPHYLOCOCCAL TOXINS AND ENZYMES

A strain of *Staph aureus* was grown in a glass tank under constant pH conditions Nutrient broth containing 1 per cent glucose was used The bacteria acted upon the glucose during growth and liberated a CO_2 which was titrated to a definite pH with KHCO_3 using an automatic pH titration assembly and thus the cultures could be maintained at any desired pH for 24 hours Analysis for the different toxins and enzymes in the culture supernatants indicated that coagulase production was optimal at pH between 7.5 and 8.0 a hemolysin was produced around pH 6.2 and hyaluronidase at pH 6.5 The production of two phosphatases was demonstrated one at pH 8.0 and another at pH 5.5 These studies emphasize the importance of pH in the production of the extracellular enzymes and toxins and the careful consideration that must be given to the pH when comparing different biologic properties of clinical strains

Bacteriology

Löfström G Hallander H O & Laurell H Department of Medical Microbiology
Division of Bacteriology University of Uppsala Uppsala PRODUCTION OF
STAPHYLOCOCCAL HAEMOLYSINS STIMULATED BY PENICILLIN IN LOW
CONCENTRATION

In previous papers (1, 2) it was shown that penicillin G and methicillin *in vitro* stimulated the production of staphylococcal haemolysins The dermonecrotic effect of staphylococci was also enhanced in rabbits which had received moderate intramuscular injections of penicillin

Some other antibiotics were tested *in vitro* Chloramphenicol acted like penicillin and stimulated in low concentrations the production of staphylococcal haemolysin Streptomycin and the tetracyclines showed no such activity

Separation on a Sephadex G 100 column was performed on supernatants of cultures which had grown in the presence of 0.1 IU/ml of penicillin (I) or without penicillin (II) It is known (3) that two active fractions appear one within 25-30 per cent of the column volume containing mainly delta lysisin the other within 45-55 per cent of the volume containing mainly alpha lysisin The experiment showed that the major part of the haemolysin activity for culture I to the first fraction for culture II to the second fraction That may indicate that the addition of the antibiotic stimulated the production of alpha lysisin while the production of delta lysisin was probably inhibited by the presence of penicillin The results are preliminary

At the VIIth International Conference for Microbiology in Stockholm 1958 Luckey (4) also reported the observation that small amounts of agents which are in larger doses to make the cells ready to an enhanced production of energy

enzymes and building material. This leads to an abnormally fast growth when the stressing agent does not exceed the bounds of toxicity. Applied on antibiotic therapy this should mean that antibiotics in low concentration might stimulate the tissue cells to enhanced activity but also give the present bacteria some stimulus. A quite delicate interaction between the cells and the bacteria is likely to occur if this theory has this general validity.

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Wielman Kristina. Central Laboratory of Clinical Bacteriology, Karolinska Sjukhuset Stockholm. COMPETITIVE INTERACTIONS BETWEEN BACTERIAL SPECIES IN EXPERIMENTAL BURNS IN THE GUINEA PIG

The intention of the experiments was to show that when bacteria of a low pathogenicity are introduced into fresh burns they can in some cases prevent or reduce colonisation by microorganisms of a higher pathogenicity which are introduced into the burns at a later stage or which occur spontaneously.

Guinea pigs were used and were given thermal full skin thickness burns in the flank corresponding in size to 2 per cent of the body area. A Dase and coagulase negative staphylococcus was taken from the animals' normal flora and applied to the burn. Similar burned but untreated guinea pigs were used as controls. *Staphylococcus aureus* (a strain which is easily identifiable because of its phage type and erythromycin resistance) was later introduced into the burn by means of a spray. In some series no such test strain was added but records were kept of *Staphylococcus aureus* spontaneously occurring in the wounds. In all series cultures taken from the wound secretions showed a statistically significant difference in both quality (the number of specimens containing *Staphylococcus aureus*) and quantity (the amount of *Staphylococcus aureus* measured by colony count). Of the specimens taken from the animals treated with the interfering strain 96 per cent contained *Staphylococcus aureus* against 60 per cent in the control animals. The amount of *Staphylococcus aureus* in the wound secretion was estimated by counting the number of colonies per plate. The average numbers were 0.4 according to a logarithmic scale. The experimental animals had an average of 0.8 against 1.9 in the control group.

Lincoln A. Department of Clinical Bacteriology, University of Göteborg, Göteborg. ANTICOPYING OF E. COLI STRAINS AND DETERMINATION OF AGGLUTININANT ANTIBODIES IN HUMAN SERUM PERFORMED IN PERSIAN PLATES

Direct bacterial agglutination was performed in perspex haemagglutination plates with 8x10 cavities. 0.05 ml of antigen suspension plus 0.05 ml of serum dilution

was incubated at 50 °C for 4 hours left at room temperature over night and read with a dissecting microscope at 16× magnification against a black background

1123 strains of *E. coli* isolated from urine were investigated with 8 antisera 545 (48.5 per cent) were agglutinated 337 (30.0 per cent) were not specifically agglutinated and 241 (21.5 per cent) were in a rough phase and agglutinated spontaneously. The specifically agglutinated strains were distributed as follows

O antigen group	02	04	06	01	07	075	018	08
Per cent of total of strains	11.7	9.8	7.0	5.6	5.3	5.1	3.0	1.4

In the serum of normal adults (blood donors and pregnant women in antenatal care) the following median titers of agglutinating coli antibodies were found

<i>E. coli</i> O antigen	01	07	04	06	07	08	014	018	075	90"
Reciprocal of titer	640	320	160	80	320	320	80	80	80	640

90" was a polyvalent antigen composed of equal amounts of the nine different monovalent O antigens. The highest and lowest titers noted were 1/2560 and 1/20. There was no difference between men and women.

Eriksen Carl & Torp 4 Institute of Clinical Bacteriology Central Hospital Malmö INACTIVATION OF CHLORAMPHENICOL RESISTANT ENTEROCOCCI

Using a disc diffusion technique for determination of bacterial susceptibility to antibiotics it was noted that sensitive staphylococci grew up to the chloramphenicol disc in the presence of enterococci resistant to the drug.

Inactivation of chloramphenicol by resistant strains has been reported for staphylococci (Chabbert & Debruge 1956) and enterococci (Miyamura 1964) but as far as known not for enterococci.

With a modified Goetz method 63 consecutively isolated chloramphenicol resistant strains of enterococci (MIC > 30 mcg/ml) were found to support satellite growth of indicator organisms in contrast to more sensitive strains (MIC < 10 mcg/ml).

The inactivation mechanism remains obscure. Dunsmoor *et al* (1963) failed to show changes in the molecular structure of chloramphenicol.

No studies seem to have been made on the clinical significance of chloramphenicol inactivation. In the laboratory it may cause false recording of resistance with the disc diffusion technique (Walworth 1966). This is especially true for cultures mixed with resistant enterococci whose colonies are easily hidden by overgrowth.

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Sjöberg I & Lindberg A 4 Department of Bacteriology, National Bacteriological Laboratory, Stockholm, SWEDEN PHAGE AND TYPING OF *STREPTOCOCCUS* SPECIES

Culture identifi ed *Streptococcus pyogenes* and *Streptococcus dysenteriae* from hospital in Sweden. The strains were typed with a set of 18 phages and from

enzymes and building material. This leads to an abnormally fast growth when the stressing agent does not exceed the bounds of toxicity. Applied on antibiotic therapy this should mean that antibiotics in low concentration might stimulate the tissue cells to enhanced activity but also give the present bacteria some stimulus. A quite delicate interaction between the cells and the bacteria is likely to occur if this theory has this general validity.

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Wickman Kristina. Central Laboratory of Clinical Bacteriology, Karolinska Sjukhuset Stockholm. COMPETITIVE INTERACTIONS BETWEEN BACTERIAL SPECIES IN EXPERIMENTAL BURNS IN THE GUINEA PIG

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Lincoln A. Department of Clinical Bacteriology, University of Göteborg, Göteborg. ON THE AGGLOMERATION OF F-COLL STRAINS AND DETERMINATION OF AGGLOMERATING ANTIBODIES IN HUMAN SERUM PERFORMED IN PERSPIRATION PLATES

Direct bacterial agglutination was performed in perspective haemagglutination plates with 8x10 cavities. 0.05 ml of antigen suspension plus 0.05 ml of serum dilution

